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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

SIR W. M. BAYLISS, F.R.S.

AND

ARTHUR HARDEN, F.R.S.

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OBITUARY NOTICE

VICTOR ALEXANDER HERBERT HUIA ONSLOW.

RARELY indeed in the history of Science can significant researches have been accomplished under conditions calling for such heroic courage as that displayed by the subject of this brief memoir. In August 1911 while still a student at Cambridge he was climbing in the Tyrol with Charles Meade the Himalayan explorer. He dived into a mountain lake, striking his head on a submerged rock. This so damaged the spine that the spinal cord was practically severed at the level of the sixth cervical vertebra, and paralysis from the waist downwards, with incomplete use of the hands and arms, was the necessary consequence.

Though never again to move from his bed Onslow faced his tragic destiny with high courage. He determined still to be of use in the world, and the moment the verdict of "incurable" was pronounced he settled down to pursuits involving not only intense intellectual activity but also scientific experimental work most of which, in spite of his grave disabilities, he carried out by himself. To this end he with immense determination compelled his partially paralysed arms and hands to the attainment of quite remarkable skill. No one ever stood at his bedside without feeling almost overwhelmed with the poignancy of what he saw. On a raised pillow the handsome intellectual face lit up with keen interest; the powerless body....

Huia Onslow, younger son of the fourth Earl of Onslow, was born on Nov. 13, 1890, at Government House, Wellington, New Zealand, while his father was Governor. He was the godson of Queen Victoria. The circumstances of his infancy were romantic. When about 12 months old he was inducted as Chief of the Ngathuia, an ancient and powerful Maori tribe. His unique Christian name, Huia, was given him in token of friendship between the British Government and the Maori race, and has reference to the Huia bird of New Zealand, of which the feathers were the tribal emblem of the Ngathuia! When fourteen years of age he again visited New Zealand and was welcomed with high festival by his tribe.

He was educated at Eton and Trinity College, Cambridge. While at the University he read natural science for one year, and then for two years turned his attention to mechanical science with the intention of qualifying for the Parliamentary Bar where he expected to find a knowledge of engineering very useful. He entered at one of the Inns of Court in 1911, but almost immediately after this, and only two months after he had qualified for his Cambridge degree, occurred the tragedy which cut him off from an active career. Active that is in the common sense; as already said there was scarcely a breach in his real

activities. During the year which followed that of his accident he read intensely, as a real student and not for pastime, the literature of a wide range of subjects—philosophy, psychology, genetics—taking the keenest interest in literary matters, in Art, and in current affairs. In this year while living in London he did experimental work in psychology, and started the Mendelian Studies upon mice and rabbits which were to continue for the rest of his life.

In the next year he started definite microscopical and chemical work on hair structure and hair pigments, a line of enquiry which always interested him, in the first instance because of its relation with his studies in genetics. At this time, as later, he used the microscope himself, and executed nearly all the manipulations connected with the instrument which stood on a little platform stretched over his bed. The chemical work was partly, but by no means exclusively, done by an assistant under his close and continuous supervision. At this time, too, he was writing much; contributing various literary articles and poems to the *Spectator* and other high class Journals.

When the war arrived it was characteristic of Onslow that he should turn his mind to possible service. He worked, not without real success, at methods for the detection of gun-fire by daylight, and also upon sound ranging. He became Joint Secretary of the Soldiers' and Sailors' Families Association for the North-western district of London. He did not however give up his scientific work at this time. He studied with S. W. Cole some of the properties of urobilin and allied pigments, and, in particular, worked at the causes of dominant and recessive whiteness in animals.

In 1915 he returned to live permanently at Cambridge and fitted up at his house a laboratory into which his couch could be wheeled. In this year a paper appeared in the *Proc. Roy. Soc.* in which he showed that Recessive Whiteness (Albinism) in rabbits is due to the absence of a tyrosinase; Dominant Whiteness on the other hand was shown to be due to the presence of an inhibitory factor. A little later there appeared in this Journal a paper on the production of the black markings on the wings of *Pieris*. Mendelian experiments with Lepidoptera were now started and continued throughout his life. Many important and beautiful results were obtained which are described in a series of papers in the *Journal of Genetics*. He worked further with S. W. Cole on the metabolism of bacteria.

About 1917 he became interested in protein chemistry and made himself an expert dealer with amino acids. Onslow was particularly anxious to solve the elusive problem presented by the quantitative separation of tryptophane and was near to its solution at the time of his death. The results of this investigation though not quite final will be shortly published. Work which is remarkable in itself, but exceptionally remarkable because of the untoward circumstances under which the skill requisite for its accomplishment was attained, is described in the paper "On a Periodic Structure in many Insect Scales and the Cause of their Iridescent Colours" which appeared in the *Philosophical Transactions* for 1921.

It is impossible in a brief notice to do full justice to his scientific work or to his other activities. The latter indeed were diverse enough to the end. He was for instance an authority on gems, and on jewellery, which he designed, making his own drawings and paintings.

In 1919 he took a step which added greatly to the happiness of his remaining years. He had indeed the gift of exacting happiness from his so grudging destiny. He married Muriel Wheldale; one whose scientific work is very familiar to the readers of this Journal.

By a pleasant and acceptable fiction Onslow claimed to be a member of the Biochemical Department at Cambridge. It became less a fiction after his marriage, for his wife brought his thoughts to us, and reported our activities to him. Indeed, after all, it was no fiction. We knew always that a short way off a colleague was daily conquering difficulties compared with which our own could be as nothing. How could we fail to work the better for that knowledge? Onslow died June 27, 1922. The tale of his years was—thirty-one!

F. G. H.

PAPERS PUBLISHED.

1. "The French Commission on Depopulation." *Eugenics Review*, 1913.
2. "Depopulation and Eugenics." *Eugenics Review*, 1913 and 1914.
3. "Hairs and Hair Pigments." *Knowledge*, 1914.
4. "Some White Ruthenian Folk-Songs," 2 parts, with Miss Iwanowska. *Folk-lore*, 1914.
5. "A Contribution to our Knowledge of the Chemistry of Coat Colour in Animals and of Dominant and Recessive Whiteness." *Proc. Roy. Soc.* 1915.
6. "On the Formation of Hair Pigment." *Knowledge*, 1915.
7. "The Cause of Albinism and Dominant Whiteness." *Knowledge*, 1915.
8. "On the Development of the Black markings on the Wings of *Pieris brassicae*." *Biochem. J.* 1916.
9. "On a substitute for Peptone and a Standard Nutrient Medium for Bacteriological Purposes," with S. W. Cole. *Lancet*, 1915.
10. "On a rapid Method for the cultural Differentiation of the Typhoid and Paratyphoid Bacilli A and B," with S. W. Cole. *Lancet*, 1916.
11. "A Note on certain names recently applied to sable mice." *Journal of Genetics*, 1917.
12. "Note on the nature of the Growths in Colloidal silica solutions." *Proc. Roy. Soc.* 1918.
13. "The Inheritance of Wing Colour in Lepidoptera. I. *Abraxas Grossulariata* var. *lutea*." *J. Genetics*, 1919.
14. II. "Melanism in *Tephrosia consonaria*." *J. Genetics*, 1919.
15. III. "Melanism in *Boarmia consortaria*." *J. Genetics*, 1920.
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I. SOME NEW REACTIONS FOR THE DETECTION OF STEROLS.

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Observations made in relation to the colour reactions in vogue for the detection of sterols have indicated that in their general character all or most of these reactions are in essence similar, and have led to the discovery of several new reactions which present certain interesting features including a greater degree of delicacy than that possessed by reactions now employed. In what follows, first three new reactions for sterols and a reaction for sterolins are described, and then some considerations are brought forward concerning the mechanism of the colour reactions shown by sterols.

Reaction A. To 2 cc. of a chloroform solution of the sterol (containing preferably 1–2 mg. of the sterol) are added 2 cc. of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportions 50 vols. : 1 vol., and the contents of the test-tube are then shaken. When the layers have separated, the upper, chloroform layer is seen to be cherry-red in colour, and the lower, sulphuric acid layer to be brownish-red in colour and to show an intense green fluorescence. The upper layer is then poured off into a dry test-tube and treated with two or three drops of acetic anhydride; as a result it assumes a bright blue colour, which lasts for a considerable time, but passes into a green within, say, an hour.

The reaction is much more sensitive and striking than the Salkowski [1872] reaction, of which it may be regarded as an elaboration. In the Salkowski reaction, a chloroform solution of a sterol is shaken with an equal volume of conc. sulphuric acid; when the liquids have separated, the chloroform layer is seen to be brownish-yellow, and the sulphuric acid layer to be yellow-brown with a green fluorescence; on allowing the test-tube to stand for several hours, the sulphuric acid layer becomes deeper and redder in colour and more strongly fluorescent, while the chloroform layer assumes, if the sterol was present in sufficient amount, a cherry-red or purple colour. Reaction A described above has several advantages over the just-described Salkowski reaction, namely: (*a*) the cherry-red colour is obtained immediately in the former, whereas in the latter it is necessary to wait several hours for its appearance; (*b*) the former reaction is given by a much smaller quantity of the sterol than the latter (*vide infra*); (*c*) the coloured chloroform solution obtained in the

former is converted by acetic anhydride into a comparatively lasting blue, whereas when the cherry-red solution obtained in the latter is treated with the same reagent a blue stage, if recognisable, is very transient, and does not add a well-defined feature to the reaction.

A comparison of the degree of delicacy of Reaction A and the Salkowski reaction showed that the former is more than ten times as sensitive as the latter.

2 cc. of cholesterol solution were used in each of the tests made in this connection. When 0·01 mg., *i.e.* 1 part in 100,000 parts, of cholesterol was present, in Reaction A a faint fluorescence could be seen in the sulphuric acid layer on allowing the contents of the test-tube to stand or on warming them, athough no colour could be seen in the chloroform layer. This amount of sterol represents approximately the limit at which Reaction A gives any result. The Salkowski reaction gave no result with this amount. When 0·1 mg. sterol was present, Reaction A gave a comparatively deep reddish-brown colour and a very marked fluorescence in the sulphuric acid layer, and, if the contents of the test-tube were heated, a barely discernible purple colour in the chloroform. When the same amount of sterol was present, the Salkowski reaction gave no colour in the chloroform and no colour or fluorescence in the sulphuric, although a faint colour and fluorescence developed in the latter if the contents of the tube were heated and then allowed to stand for about ten minutes. Even when 1 mg. of sterol was present, a cherry-red colour in the chloroform was not usually noticeable in the Salkowski reaction; any faint colour which might possibly have developed during the night having disappeared by the morning. With 1 mg. of sterol, Reaction A gave an immediate cherry-red colour in the chloroform.

Reaction A was applied with positive and essentially identical results to three samples of cholesterol and five samples of phytosterol[1]. In this reaction, amyrin gives in the chloroform no colour but in the sulphuric acid an orange-red colour (which soon becomes blood-red) and a deep green fluorescence; abietic acid gives nothing very characteristic: the chloroform becomes merely pale brown and the sulphuric acid dark red-brown and slightly fluorescent.

A modification of the Salkowski reaction, made by Hesse [1881], and consisting in using sulphuric acid of specific gravity 1·76 instead of 1·84, has the advantage over the Salkowski reaction of giving a characteristic colour (rose) in the chloroform immediately, but it has the disadvantage of not giving fluorescence in the acid and of being less sensitive than the ordinary Salkowski reaction. It must be considered as less generally useful than either the Salkowski reaction or Reaction A.

[1] These samples were as follows: (*a*) a sterol from the resin of *Hevea* rubber [Whitby and Dolid, 1921]; (*b*) phytosterols from *Adonis vernalis*; (*c*) sitosterol from *Echinacea* root; (*d*) and (*e*) preparations of sitosterol from wheat. The author is much indebted to Dr F. W. Heyl, the Upjohn Co., Kalamazoo, Mich. for samples (*b*) and (*c*), and to Prof. L. Kahlenberg, University of Wisconsin, for samples (*d*) and (*e*).

Reaction B. To 2 cc. of a solution of sterol in glacial acetic acid, containing conveniently 0·2–0·5 mg., are added with shaking 25 drops of a reagent prepared by mixing conc. sulphuric acid and formalin in the proportion 50 vols. : 1 vol. The result is a rose-coloured and fluorescent solution.

The rose colour, which has a tinge of purple, is not permanent, but on standing changes to yellow-brown. When only 0·01 mg. of sterol is present, the rose colour changes quickly, but more usually (*i.e.* with an amount of sterol of the order first mentioned) no change in the full, initial brightness of the colour is apparent for about two minutes, and the change to brown is not complete until an hour or more has elapsed.

This reaction is more sensitive than any colour reaction hitherto proposed for the detection of sterols. It is approximately twice as sensitive as the Liebermann-Burchard reaction [Liebermann, 1885; Burchard, 1889], although, unlike the latter, it is not suited to the quantitative determination of sterols. In Reaction B colour is just recognisable with 0·005 mg. of sterol per cc., *i.e.* the limit of sensitiveness of the reaction is 1 in 200,000. With 1 mg. per cc. the colour developed is too deep to allow one to see through the solution in a test-tube. A suitable concentration at which to apply the reaction is 0·1–0·25 mg. sterol per cc.

The reaction may be applied to an ethereal instead of an acetic acid solution of a sterol; but in this connection it should be noted that on standing for several hours a blank test will show a brown colour.

In Reaction B amyrin gives a cherry-red, strongly fluorescent solution, which becomes brown on long standing; abietic acid, a not very characteristic green-brown colour[1].

Reaction C. A few milligrams of a sterol are added to one drop of acetic anhydride on a piece of porcelain and gently heated until it has melted and excess of anhydride has been driven off. The melt is allowed to cool completely, and is then moistened with conc. nitric acid. Within a few seconds the material assumes a blue or blue-green colour.

This reaction is chiefly of value for cholesterol; it is of less value for phytosterols. Five samples of phytosterol[2] to which the reaction was applied behaved somewhat differently from cholesterol: even when the reaction was carried out with great care (the melt being distributed in as thin a layer as possible), they gave a much less intense colour than did cholesterol. The difference in behaviour of cholesterol and phytosterols in this reaction is not, however, sufficiently sharp to allow of the reactions being used as a means of distinguishing animal and vegetable sterols.

[1] The behaviour of amyrin and abietic acid in certain other sterol colour reactions may be put on record here. In Tschugajeff's reaction (see later) amyrin gives a red, which is somewhat browner than that given by a sterol and is unaccompanied by fluorescence; abietic acid gives a still browner red which is, however, accompanied by fluorescence. In Lifschütz's reaction (see later) neither substance gives a characteristic colour but merely a pale brown colour.

[2] Described in footnote, p. 6.

The author could not confirm the statement of Kahlenberg [1922] that arsenic chloride provides a means of distinguishing cholesterol from phytosterols by yielding a cherry-red solution with the former and a colourless solution with the latter. Each of the five samples of phytosterol referred to above gave a cherry-red solution in arsenic chloride.

A fusion test for the detection of cholesterol has been described by Obermüller [1891], but does not appear to be very useful, as it would seem to demand exceptionally dry samples of cholesterol for success. The present author obtained negative results on applying Obermüller's reaction to samples of cholesterol taken directly from stock bottles.

Sterolin reaction. Sterolins[1] give most of the colour reactions of sterols (*vide infra*). The following reaction, which depends in part on the sterol portion and in part on the glucose portion of the molecule, has been designed in conjunction with Mr J. Dolid, for recognising sterolins and at the same time distinguishing between sterolins and sterols.

1–2 cc. of conc. sulphuric acid is poured on to a few particles of a sterolin contained in a test-tube, and the mixture is warmed gently until the solid has gone into solution; the liquid is then cooled, and a cold, saturated, aqueous solution of thymol is poured on top of it. The result is an orange colour and a strong green fluorescence in the lower layer and a violet ring at the junction of the upper and lower layers. On allowing the test-tube to stand, the violet colour tends to spread through the upper layer.

When the reaction is applied to a sterol, a lower layer similar to that described above is obtained, but a violet ring is missing.

The sample of sterolin to which the above reaction was applied was isolated from the resin of *Hevea* rubber [Whitby and Dolid, 1921]. Its behaviour in the various sterol colour reactions was as follows. It gave Reaction B, the Liebermann-Burchard, the Tschugajeff, and the Lifschütz reactions. In Reaction A its behaviour was slightly different from that of a sterol, due doubtless to the circumstance that it is only very slightly soluble in chloroform: fluorescence occurred in the sulphuric acid layer, as in the case of a sterol; the colour in the chloroform layer was not, however, as in the case of a sterol, cherry-red, but a brownish yellow, which soon deepened to a red-brown; and, when the chloroform layer was poured off and treated with acetic anhydride, it became blue, but the blue remained for a shorter time than in the case of a sterol.

A consideration of the colour reactions applicable to the detection of sterols in solution indicates the existence of a general similarity between these superficially different reactions. In all or nearly all the reactions in question three points in common are discernible, namely, (*a*) the dehydration of the sterol

[1] The name phytosterolins has been given by Power and Salway [1913] to glucosides of phytosterols, such as have been recognised during recent years in a considerable number of plants.

molecule with the production of a colourless substance, (*b*) the appearance of a coloured product by the interaction of this first substance with a second (coupling) substance derived from the sterol or introduced in carrying out the reaction, (*c*) the use of an agent for rendering the medium anhydrous. As is pointed out below, the first substance is probably a hydrocarbon—*e.g.* a cholesterylene or cholesterylin—formed by the withdrawal of the elements of water from the sterol. The dehydrating agent responsible for its production may or may not also be the agent used to render the medium anhydrous. The coloured products obtained in the sterol colour reactions are very sensitive to traces of moisture. The actual colour obtained depends upon both the thoroughness with which the medium is dehydrated and the nature of the second (coupling) substance. These points can be illustrated by considering some of the individual reactions.

The Salkowski reaction. If the cherry-red chloroform layer is poured into another test-tube, it is usually seen to lose its colour as a result of the transference. That the destruction of the colour is due simply to the small amount of moisture present in a nominally dry test-tube is shown by, among other observations, the fact that the colour can be restored, not only by conc. sulphuric acid (as was noticed by Salkowski), but also by phosphorus pentoxide.

In the Salkowski reaction the sulphuric acid acts both to produce the first, colourless substance by its action on the sterol and to render the chloroform anhydrous. The presence, immediately after the introduction of the sulphuric acid, of the first, colourless substance, capable, by reacting with a second substance, of giving a coloured product, is shown by the observation that, if the chloroform layer is poured off at once, before a cherry-red colour has developed, and is then treated with formaldehyde[1], which serves as the coupling substance (cf. Reaction A, *infra*), a cherry-red or purple colour is obtained at once. In the ordinary Salkowski reaction a second (coupling) substance apparently arises only slowly. The rate at which the second substance necessary for the appearance of colour arises seems to be different in different solvents, and is, for example, noticeably slower when chlorobenzene is used as a solvent for the sterol than when chloroform is used.

Reaction A. In the reaction the first substance couples at once with formaldehyde to give a cherry-red product. Unlike the paler cherry-red colour produced in the ordinary Salkowski reaction, the colour produced in Reaction A is not destroyed by pouring the chloroform layer into another test-tube. Acetic anhydride changes the colour through purple and then through blue to green. The characteristic feature here is the comparative persistence of the blue stage, which has hitherto been discernible in sterol colour reactions (*e.g.* in the Liebermann-Burchard reaction) only as a very fleeting stage. By choosing a suitable solvent, in which the blue colouring matter is insoluble, the latter can be actually isolated as a solid.

[1] In, say, chloroform or ethereal solution.

Two drops of conc. sulphuric acid were added to 3 cc. of a solution of cholesterol in ethyl bromide. The solution immediately took on an orange colour (which became blood-red within a few minutes) and appeared fluorescent. A small amount of a brown solid separated in flocks. The blood-red solution was treated with a few drops of acetic anhydride, and then allowed to stand for half-an-hour. While standing, the solution passed through a purple stage, then became blue, and gradually deposited a blue solid. That this solid represents the blue product seen in solution in various of the sterol reactions, is indicated by the fact that it was found to be soluble in chloroform, and, when dissolved in the latter, to suffer a change of colour to green on treatment with acetic anhydride.

Reaction B. Here, as in the previous reaction, sulphuric acid both dehydrates the medium and acts on the sterol to produce the first, colourless substance, and the latter then reacts with formaldehyde to give a coloured product. This reaction is more sensitive than Reaction A because of the miscibility of the sulphuric acid with the solvent. In the case of the Salkowski reaction and Reaction A some of the colour-producing substance goes into the sulphuric acid layer. In this connection the following observations may be noted.

(1) That, alike in the Salkowski reaction and in Reaction A, the same substance is in question in the two layers, is indicated by the observation that formaldehyde, which, as already mentioned, intensifies the colour in the chloroform, also intensifies the depth of the colour and the fluorescence in the sulphuric acid.

(2) If in Reaction A the two layers are shaken together vigorously, the upper layer can be rendered almost colourless, owing to the transference of colour to the sulphuric acid layer.

(3) If conc. sulphuric acid is dropped into a solution of cholesterol in carbon tetrachloride, the latter assumes at once, as the acid drops through it, a deep yellow colour, and the sulphuric acid forms a colourless layer at the bottom; if now the two layers are shaken together well, the colour goes almost completely into the sulphuric acid layer, the upper layer becoming nearly colourless.

In connection with carbon tetrachloride, it is interesting to note that the purple-coloured product obtained in various of the sterol colour reactions is insoluble in this solvent and can be isolated by its use.

(*a*) The above-mentioned yellow, carbon tetrachloride layer was poured off and treated with acetic anhydride. A dark-purple solid gradually separated, the liquid itself finally becoming green. The solid was removed, and was found to be soluble in warm chloroform. The purple chloroform solution of the solid, when treated with acetic anhydride, became converted through blue to green.

(*b*) The yellow carbon tetrachloride solution was treated with formaldehyde. There separated a purple-coloured solid with properties similar to those of the purple solid just mentioned.

It thus appears that the purple solid, mentioned under (*a*), and obtained by treating the first, colourless substance in carbon tetrachloride with acetic anhydride, is essentially identical with the purple or cherry-red substance obtained by treating the first colourless substance with formaldehyde.

Liebermann-Burchard reaction. In this reaction [Liebermann, 1885; Burchard, 1889] a green colour is produced by the addition of a drop of conc. sulphuric acid to a solution of a sterol in acetic acid containing some acetic anhydride. The sulphuric acid produces the first, colourless substance from the sterol, and the acetic anhydride then reacts with this substance to give a purple-coloured product, which it then changes to a blue and finally to a green product. The acetic anhydride also serves to dehydrate the medium. The power of acetic anhydride to change the colour of the earlier coloured stages and at the same time to dehydrate the medium, is shown in the following observations.

(*a*) The cherry-red chloroform layer obtained in Reaction A was poured off and diluted with chloroform from a stock bottle until it became colourless. (The amount of moisture in an ordinary sample of chloroform is sufficient to discharge the colour.) Acetic anhydride was then added. The result was to produce a blue colour (which later turned green), just as it would have been had the colour not first been discharged.

(*b*) To a blue chloroform solution obtained in Reaction A drops of water were added until the colour was discharged. (The colour could be restored by adding phosphorus pentoxide.) A little acetic anhydride was then added. The result was a green colour.

Tschugajeff reaction. In this reaction [Tschugajeff, 1900] an eösin-red colour is obtained by boiling an acetic acid solution of a sterol with zinc chloride and acetyl chloride[1]. The zinc chloride acts both to withdraw water from the sterol molecule and to dehydrate the medium. In the latter function it is doubtless assisted by the acid chloride.

It was found that if an acetic acid solution of a sterol is boiled with powdered, dry zinc chloride, without the addition of an acid chloride, a purple colour develops. Hence it would appear that zinc chloride alone is capable of producing from a sterol both a first and a second substance, which together give rise to what has previously been referred to as the first coloured stage (cherry-red, purple, or rose). As in the case of the Salkowski reaction, the amount of the second substance formed is apparently insufficient to lead to the full development of colour which the amount of the first colourless substance produced is capable of yielding; for, if a little formaldehyde is introduced (as vapour) into the solution, the purple colour obtained on boiling is deepened.

Lifschütz reaction. In this reaction [Lifschütz, 1898] benzoyl peroxide serves to dehydrate the medium and to react with the first, colourless substance, formed from the sterol by the action of sulphuric acid.

It would appear that the hydrocarbons—*e.g.* cholesterylenes and cholesterylins—which can be obtained from sterols by the action of sulphuric acid

[1] It was found that the acetyl chloride may, with identical results, be replaced by benzoyl chloride.

or zinc chloride, and have been studied by Zwenger [1848, 1849] and by Mauthner and Suida [1896], are concerned in the reactions discussed above.

A mixture of hydrocarbons, prepared from cholesterol according to the procedure of these authors, was dissolved in chloroform. The solution thus obtained was colourless, but when warmed with phosphorus pentoxide became rose- or cherry-red. If a little formaldehyde vapour was passed into the solution before the addition of the pentoxide, a deeper purple colour was obtained. If the purple solution was poured off and treated with acetic anhydride, its colour changed through blue to green. These results are strictly parallel with those observed in connection with the sterol colour reactions.

It appears that in the mixture of hydrocarbons (? with possibly other substances) which sulphuric acid produces from cholesterol there are present both the substances previously referred to as the first, colourless substance and the second (coupling) substance; as it is sufficient to dissolve the mixture in a thoroughly dehydrated medium for a cherry-red colour to make its appearance. It appears, further, that, as was seen to be the case in the Salkowski and Tschugajeff reactions, here too an insufficient amount of the second (coupling) substance is present; as the introduction of formaldehyde deepens the colour obtained.

Summary.

Three new colour reactions (A, B and C) for the detection of sterols are described. Reaction A is more striking and sensitive than the usual chloroform-sulphuric acid reaction. Reaction B is more sensitive than any sterol colour reaction hitherto described.

A reaction is described for detecting sterolins and distinguishing them from sterols.

In a general discussion of the reactions applicable to the detection of sterols in solution it is concluded that all or almost all such reactions are, in their essential features, similar.

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II. STUDIES IN EXPERIMENTAL TETANY.

I. ON THE DISTRIBUTION OF CALCIUM IN THE PLASMA AND CELLS.

II. ON THE VARIATIONS IN COLLOIDAL AND IONIC CALCIUM.

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I.

Theories put forward with regard to the causation of tetany.

Although much research work has been done and a great deal written on this subject, it may not be out of place here briefly to recapitulate the main theories which have been put forward in an attempt to explain the chemical changes occurring in the blood and their relationship to the symptoms following a loss of function of the parathyroid glands.

The first hypothesis formulated was that the condition was due to a disturbance of the metabolism of calcium salts whereby the tissues and body fluids were deprived of soluble calcium, hence the irritability of the nervous system. This was based upon work of MacCallum [1902–1904] and Loeb [1902, 1, 2; see also 1915] in 1902 and upon researches by Sabbatani who showed that salts of bivalent metals have a depressing effect on the central nervous system, and that such salts counteract the effect of calcium-precipitating substances on the cerebral cortex.

Further confirmation of this theory was forthcoming in the work by Quest [1906] upon the calcium content of cerebral tissue in tetany and by the demonstration of the curative effect of calcium salts upon tetany by MacCallum and Voegtlin [1909]. These workers had previously clearly shown that tetany was associated with a deficiency of calcium salts. That the injection of calcium salts is efficacious in the treatment of tetany in children has been more recently confirmed by Howland and Marriott [1916].

In 1915 Wilson, Stearns and Thurlow [1915] brought forward evidence to the effect that after parathyroidectomy a condition of alkalosis may develop, which condition is neutralised by acid products formed by the muscular activity associated with the muscle tremors incident to tetany.

That the condition preceding an acute attack of tetany is one of alkalosis, they deduced from a study of the variations in the values of the dissociation constant of oxyhaemoglobin, the alveolar carbon dioxide pressure, and the

hydrogen-ion concentration of the blood of dogs subjected to parathyroidectomy. They further showed that the condition of acidosis following several attacks of tetany and the injection of a mineral acid are associated with relief from tetany. In this condition of acidosis the dissociation constant of oxyhaemoglobin is lowered as is also the alveolar carbon dioxide pressure.

Wilson, Stearns and Janney [1915] also indicate that there is a sudden diminution in the excretion of acids and ammonia and a decrease in the ammonia ratio and the hydrogen-ion concentration of the urine immediately following parathyroidectomy. With the onset of tetany there is an increased elimination of acids and ammonia with an increase in the hydrogen-ion concentration value of the urine.

The third hypothesis which has been brought forward is that the condition of tetany is due to the circulation of some toxin or toxins produced by the breakdown of body protein and to be found in the blood and urine of the patient. This theory was the outcome of work by Noël Paton, Findlay and Burns [1915, 1916] and was first published in 1915.

The idea that some product of muscle protein decomposition was the responsible factor was not then entirely new, it having been suggested by Pekelharing and Van Hoogenhuyze [1910] some five years previous to the publication of the researches of Paton and his co-workers.

It is well known that the injection of guanidine sulphate or methylguanidine [Paton, Findlay and Burns, 1915] gives rise to symptoms of hyper-irritability of nerves and it is to Koch [1912] that the credit is due for first discovering the presence of methylguanidine in the urine of dogs suffering from parathyroid tetany.

The distribution of Calcium in the plasma and cells.

Numerous estimations upon the total calcium of blood, of plasma and of serum have been carried out, and while a certain divergence of results exists, the general average of results arrived at by various workers is practically the same. In their earlier work MacCallum and Voegtlin stated that the per cent. of calcium in whole normal blood was about 13·3 mg. while in tetany blood it was about 5·4 mg. Several years later these workers by means of a different analytical method arrived at the conclusion that their former results were too high and that more correct figures for the percentage of calcium in normal and tetany blood, would be 6·1 mg. and 2·7 mg. respectively.

Variations in results are undoubtedly due to difference in the methods used for the estimation of blood calcium. Of the two methods more generally used in recent years, namely those of Lyman [1917] and Halverson and Bergheim [1917], it seems that the experimental error is much more under control with the former than with the latter. A nephelometric method which depends upon the accurate matching of shades or depth of colour, always introduces a personal error, and such a method cannot safely be adopted until by practice he who uses it is assured by stringent tests that his readings

are within 0·5 to 1 mm. of the correct figure. The latter method is one of titration with potassium permanganate, a titration in which the end point is rather difficult of determination and one in which one drop of the permanganate solution too many or too few may result in an error of ± 5 %.

In the present investigation Lyman's method has been used and has proved very satisfactory for estimating small amounts of calcium. The nephelometer used was made by taking a Duboscq colorimeter, and replacing the cups and plungers with hard rubber cylinders into which short prepared test-tubes fitted accurately. The test-tubes were supported by means of their flanged edges resting in two lateral slits cut into a hard rubber plate which was screwed on to the colorimeter beneath the prism. The cylinders, supported by movable rings resting on the cup supports, were so adjusted that when the tops were in contact with the hard rubber plate, thereby excluding all light, the indicators on the millimeter scale stood at the zero mark.

The nephelometer-colorimeter attachment or illumination box was made of wood painted black on the outside and white within, the partition placed between the lamps: the colorimeter contained a plate of frosted glass, and the illumination consisted of one set of two 100 c.p. 110 volt lamps and one set of two 100 c.p. 220 volt lamps, so arranged that any number could be used for illumination.

In determining the distribution of calcium in plasma and cells, it was necessary to obtain accurate values for the proportion of cells to plasma in whole blood. For this purpose it was found that the ordinary haematocrit was not satisfactory and the following method was adopted. A piece of pressure glass tubing 10 cm. long having an internal diameter of 1 mm. and a wall thickness of 3 mm. was taken and the ends made smooth with a file.

To a 50 cc. metal centrifuge holder a small right-angled piece of iron was attached so that it formed an inverted L, the arm of the letter being placed over the mouth of the cup and having a tapped hole at the distal end, placed accurately over the centre of the cup. The glass tube filled with blood was placed in a stout rubber band, a small brass cup was placed over the upper end of the tube the lower end of which was then pressed firmly into the rubber cushion in the bottom of the centrifuge cup, by means of a nut which passing through the tapped hole fitted into a narrow and comparatively deep hole in the brass cup.

This device (Fig. 1) was used in duplicate and after centrifuging the specimen of blood for 15 minutes very accurate measurements could be made of the proportion of cells to plasma in a length of 10 cm. of blood.

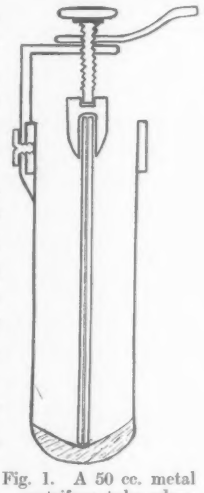

Fig. 1. A 50 cc. metal centrifuge tube adapted for obtaining haematocrit readings with a 10 cm. length of blood.

Table I. *The distribution of calcium in the cells and plasma of normal dogs.*

Dog	p_H	Total Ca mg. %	Haematocrit reading mm.	Plasma Ca per 100 cc. blood	Cell Ca per 100 cc. blood	Cellular Ca % of total
I	7·5	9·60	3·7	8·36	1·24	12·19
II	7·5	8·1	3·6	7·20	0·90	11·1
III	7·5	8·00	8·4	7·26	0·74	9·3
IV	7·5	8·00	2·8	7·28	0·72	9·0
V	7·55	7·75	3·4	6·79	0·96	12·2
VI	7·55	8·10	3·1	7·24	0·86	11·6
VII	7·55	10·00	3·1	8·83	1·17	11·7
VIII	7·50	8·20	3·2	7·8	0·40	4·9
IX	7·4	7·50	3·2	7·20	0·30	4·0
X	7·5	7·85	3·5	6·95	0·90	10·2
XI	7·45	9·12	3·5	8·25	1·87	9·6
XII	7·45	9·60	3·0	8·40	1·20	12·5
XIII	7·45	10·80	2·7	9·56	1·24	11·5
XIV	7·5	11·00	3·4	9·90	1·10	10·0
XV	7·5	9·70	2·8	8·85	0·85	8·8
XVI	7·5	10·00	2·5	8·70	1·30	13·0

Table II. *The distribution of calcium in the cells and plasma of tetany dogs.*

Dog	p_H	Total Ca mg. %	Haematocrit reading mm.	Plasma Ca per 100 cc. blood	Cell Ca per 100 cc. blood	Cellular Ca % of total
I	7·6	5·82	3·0	5·32	0·50	8·5
II	7·70	5·37	1·9	4·86	0·51	9·5
III	7·65	5·62	2·8	5·19	0·43	7·6
IV	7·75	5·75	3·2	5·16	0·51	10·26
V	7·6	6·5	8·5	6·04	0·46	7·1
VI	7·7	6·25	3·3	5·83	0·42	6·7
VII	7·6	6·50	3·6	6·1	0·40	6·2
X	7·65	4·93	3·5	4·45	0·48	9·7
XI	7·65	4·75	2·7	4·41	0·34	7·1

From Table I it will be seen that the average result of the total calcium of the blood of 14 dogs is 9·12 mg. %. In computing this average the figures for dogs Nos. VIII and IX are not included as these dogs were suffering severely from mange.

The average figure for the amount of calcium in plasma from 100 cc. of blood is 8·11 mg. while for the corpuscles from the same amount of blood it is 1·01 mg. that is to say in a hundred parts by volume of blood we find a distribution of calcium between the plasma and the cells in the proportion approximately of 8 to 1.

In a series of some twelve rabbits in which similar estimations were made it was found that the figures were slightly higher, being for whole blood 10·0 mg. %, for plasma from 100 cc. of blood, 8·6 mg. and for the cells 1·4 mg.

Estimations were performed upon the parathyroidectomised dogs usually on the third day after operation. If the animals survived for two or three days longer, a second determination was made. On several of these animals a partial parathyroidectomy was performed but no conclusive results were obtained immediately following such an operation and the results quoted are those in totally parathyroidectomised animals. The p_H was estimated on the

day following operation on blood drawn from the left ventricle, the method used being that of Levy, Rowntree and Marriott [1915]. The alkaline reserve was determined by the colorimetric method of Marriott [1916]. The relation of the p_H to alkaline reserve will form the subject of a further communication in reference to the carbon dioxide combining power of the plasma of tetany dogs.

Average figures on seven completely parathyroidectomised dogs quoted in Table II show a whole blood calcium content amounting to 5·72 mg. %, with a plasma and cell content per 100 cc. of whole blood of 5·26 mg. and 0·46 mg. respectively. It is thus seen that in experimental tetany the ratio of plasma calcium to cell calcium is approximately 11·5 to 1·0.

Table III. *A comparison of the average figures for calcium distribution in normal and tetany dogs.*

Ca % in mg. whole blood	Plasma Ca mg. per 100 cc. of blood	Plasma Ca % of total Ca	Cell Ca mg. per 100 cc. blood	Cell Ca % of total Ca	Remarks
9·12	8·11	88·9	1·01	11·1	Normal dogs
5·72	5·26	91·9	0·46	8·1	Tetany dogs
37·4	35·2	—	54·4	—	Percentage fall in calcium

The alteration in the distribution may be more clearly seen from Table III, in which the relative percentage of calcium in the plasma and cells to the total blood calcium is shown and in which the comparative alterations are indicated as percentages of the normal contents. It is here shown that in the condition of tetany there is a considerable loss of cell calcium amounting to 54·4 %, as compared with a plasma loss of 35·2 %.

II.

Observations on diffusible and colloidal calcium in normal and parathyroidectomised dogs.

In 1921 a method for the estimation of diffusible calcium was introduced by von Mysenbug and his colleagues [1921]. This method is one of compensation dialysis, in which the diffusible salt in the dialysee is balanced by the addition of an equal amount of the salt to the dialysing fluid.

The separation by filtration of the diffusible salts of blood serum can be effected by subjecting the filter to a pressure of 150 mm. of Hg. This method was employed by Cushny [1920], but to determine accurately the amounts of a salt in ionic and colloidal form, it is essential not to alter the balance between them by removal from the solvent of the diffusible salt. According to the law of mass action the removal of the ionic calcium would result in a further breakdown of calcium in combination in order to restore the normal balance. For this one reason therefore apart from others the compensation method of dialysis is the better and has been made use of in this work.

There are however difficulties associated with the method, the chief of which is the phenomenon of negative osmosis, a phenomenon in which water

may pass from the more concentrated to the more dilute side, that is against osmotic pressure. It may be mentioned here that in dialysing blood serum it is not always sufficient that the dialysate be a solution isotonic with blood serum.

Alterations in permeability of the membrane may be brought about by alterations in the membrane itself, by changes in the solutions within or without the membrane, and by changes in the electrical double layer on the surface of the membrane. If a solute of opposite electrical sign come within the sphere of electrical attraction of the membrane it will be adsorbed. If the adsorbed material be an amphoteric colloid the electrical charge on the membrane will be modified. It is this adsorption by membranes of amphoteric colloids which is responsible for the phenomenon of negative osmosis; water therefore will pass through the membrane at a rate depending upon the density, not the sign, of the electrical charge on the membrane.

The diffusion may be caused either by pure osmosis or by electrical osmosis, the latter being dependent upon the presence of electrolytes and also upon the fact that collodion membranes adsorb amphoteric colloids, thus producing electrical charges on the membrane.

Such salts as oxalates, citrates and phosphates attract water markedly but the effect of the anion may be neutralised by the cation, and this explains the weak action of calcium chloride where the electrical charge on the cation practically neutralises the two charges on the anions.

The difficulties associated with semi-permeable membranes are well known [see Burns, 1921 and Loeb, 1917].

It has been found that very thin sacs made of parlodion are too readily permeable to protein while sacs which are too thick are useless because of negative osmosis whereby the volume of the serum is increased. In such sacs the degree of permeability to electrolytes is also considerably reduced. In a very interesting paper on the degrees of permeability of collodion sacs Eggerth [1921] has shown that the speed of diffusion of electrolytes, "non-dialysable" colloids, like congo red, and proteins of serum and of blood cells depends upon the permeability of the membranes, and the relative permeability of the membrane depends upon the proportions in which alcohol and ether are present in the alcohol ether solvent used for dissolving the pyroxylin, or parlodion to give it the name under which it is manufactured. Sacs made by dissolving collodion in ether and alcohol in the proportion of 60 to 40 parts, were found to be impermeable to protein after three hours' dialysis. They still retained their maximal permeability to crystalloids, all of which were found to pass through in 15 minutes. To obtain sacs which will withstand dialysis of 24 hours at 15–20°, it is necessary to employ a "30 to 40 alcohol" membrane. With such a membrane the diffusion of electrolytes is complete in 10 to 15 minutes and it has been demonstrated that in the presence of protein the diffusion of calcium salts is complete well within 24 hours.

While a considerable drop in the total calcium of the blood and of its cellular elements has been noted in parathyroid tetany, the question as to

whether or not this was due to a loss in free or combined calcium has not been definitely settled. By dialysis we can determine the amounts of free and combined calcium in blood plasma or serum but no method has been perfected whereby a similar determination can be carried out with regard to the cellular elements of the blood.

That alterations may occur in the diffusible calcium in rickets and in tetany was suggested by von Mysenbug and his co-workers, but they in a series of four experiments found no distinct variations from the normal.

Methods.

Preparation of parlodion sacs. The shreds of parlodion are dried in the air and dissolved in ether and 95 % alcohol in the required proportions. After solution of the parlodion shreds is complete, the bottle, firmly stoppered, is allowed to stand for two weeks.

Two small test-tubes 15 mm. internal diameter and 60 mm. long are filled with the mixture, centrifuged to bring all air bubbles to the surface, the hardened surface then peeled off and the contents emptied into the stock bottle, the tube being rotated meanwhile, in order to secure uniform thickness of the wall of the sac. The tube is clamped in the inverted position for 15 minutes, then placed in cold distilled water for half-an-hour. The upper rim is loosened by means of a knife and a thin glass rod with a bulbous tip gently pushed between the sac wall and the glass to the bottom of the test-tube, which is kept half filled with water. The sac and the glass rod are withdrawn together and the sac immersed in cold distilled water.

Compensation dialysis. In view of the fact that in parathyroid tetany variations in diffusible calcium may be great or small, it was necessary to make up a series of dialysing calcium solutions in order to balance as nearly as possible, the amount of free calcium found in the serum. For this purpose therefore ten dialysing solutions were made up giving a percentage of normal serum calcium varying from 40 to 80. These solutions were made from a stock solution of calcium chloride of about 1 % strength, the amount of calcium in the stock solution having been determined by the nephelometric method. The following is the series of solutions employed:

Compensation dialysing solutions.

Solution	Amounts of Ca in mg. in 2·5 cc.		Amounts of Ca in mg. %		% of normal Ca
	Estimated	Calculated	Estimated	Calculated	
A	0·206	0·193	8·25	7·71	39·28
B	0·213	0·210	8·54	8·40	40·65
C	0·243	0·241	9·75	9·65	46·42
D	0·257	0·262	10·30	10·50	49·10
E	0·290	0·289	11·62	11·58	55·33
F	0·312	0·315	12·50	12·60	59·52
G	0·343	0·338	13·75	13·50	65·47
H	0·367	0·363	14·68	14·70	69·90
I	0·390	0·355	15·62	14·19	74·38
J	0·406	0·420	16·25	16·70	77·38

To 2·5 cc. of the solution employed, 2·5 cc. of a double strength dialysing fluid were added the composition of which is as follows:

Dialysing fluid (Greenwald).

Salt	% amounts single strength g.	Molar strength	Amounts in 2000 cc. double strength
$NaHCO_3$	0·2520	0·003	10·08 g.
KH_2PO_4	0·0088	0·000065	12·9 cc. *M*/5
$MgCl_2$	0·01059	0·00011	0·2118 g.
KCl	0·02960	0·000397	1·184 g.
NaCl	0·6250	0·01069	25·0 g.

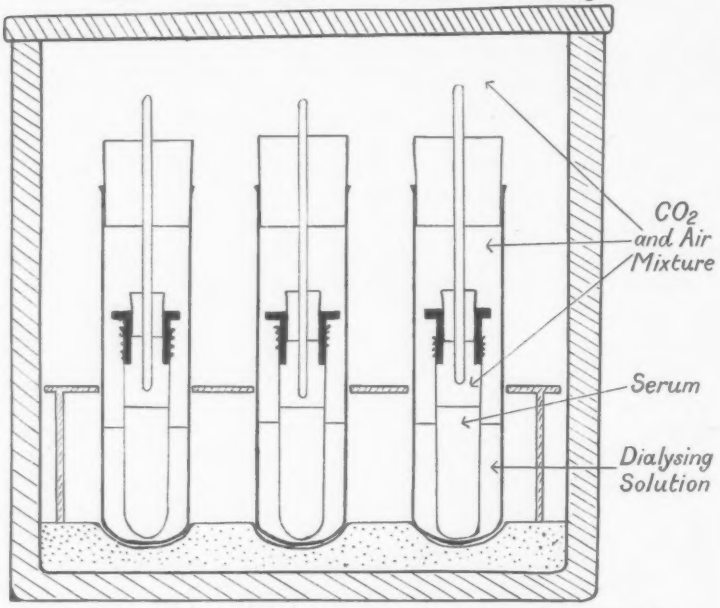

Fig. 2. Diagram of the method of setting up a series of six sacs for compensation dialysis of the blood serum.

Since the balance between diffusible and colloidal calcium is in some measure dependent upon the hydrogen ion concentration of the serum, it is essential to bring the CO_2 tension of the serum into equilibrium with an atmosphere of CO_2 of a tension approximating that of alveolar air. To do this a large spirometer was filled with CO_2 and air, these were thoroughly mixed, and the percentage of CO_2 estimated daily by the Henderson and Morriss [1917] method.

The gas was passed through a bottle containing moist glass beads then through two separatory funnels placed in series, the first containing about 20 cc. of serum the second about 10 cc. of the double strength dialysing fluid. This equilibration was carried out at room temperature which during the course of these experiments varied from 30 to 33°.

The dialysis. The sacs to be employed were thoroughly washed in distilled water, shaken free of water and allowed to drain, but not allowed to dry in the air. The test-tubes used for dialysis were $\frac{3}{4}''$ wide by 4″ long and into these were placed 2·5 cc. of the double strength dialysing fluid, and CO_2 passed into the test-tubes for a few minutes.

The sacs supported on small hard rubber cylinders, were filled with 5 cc. of serum, CO_2 was passed into the sacs, a small cork firmly inserted into the top of each cylinder, and the whole supported in the test-tube as shown in Fig. 2. Dialysis was carried out for 24 hours at a temperature of 12 to 15°.

Discussion of Results.

In the following series of Tables Nos. IV to VII, the results obtained in normal, partially and totally parathyroidectomised dogs are given.

Table IV. *Total calcium in normal, partially and completely parathyroidectomised dogs* (mg. per 100 cc.).

Normal	Partial	Complete
11·24	6·25	5·0
10·50	6·13	5·10
11·20	8·20	5·00
11·30	Average 7·04	4·70
12·70		5·20
12·60		4·60
9·00		5·40
Average 11·30		Average 4·95
100 %	→ drop 37·72 %	→ drop 56·20 %

Table IV shows the variations in percentage of the calcium content of serum under the above three conditions. From a normal average of 11·30 mg. %, the calcium fell in partially parathyroidectomised animals to an average of 7·04 mg. %, to be followed by a further fall upon complete parathyroidectomy to an average figure of 4·95 mg. %. It is thus seen that the loss of calcium from the serum amounts to 56·20 % of the whole.

With regard to diffusible calcium, the figures quoted in Table V, which comprises results obtained from a series of seven normal dogs, are comparable to those of Cushny and von Mysenbug, namely that in normal animals the diffusible calcium amounts to between 60 and 70 % of the whole calcium content of the blood taking 10·5 mg. as the percentage of calcium in normal blood serum.

In comparing the results recorded in Tables VI and VII, results from partially and completely parathyroidectomised dogs respectively, it is seen that there is no essential difference in the amounts of calcium recorded.

It is somewhat difficult to say to what extent a single parathyroid gland may or may not be functioning. The upper left parathyroid was left with a small piece of thyroid tissue. In all of these cases the gland retained was distinctly identified, but we must admit that this method of leaving a gland

RESULTS OF COMPENSATION DIALYSIS OF SERUM CALCIUM.

NORMAL DOGS.

Table V.

Dog I. p_H 7·5; Total Ca = 10·5 mg. %; Ca in 5 cc. serum = 0·525 mg.; CO_2 tension = 49·64 mm. Hg.

Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium	Compensation dialysing solution
Begin	End	Begin	End	%	
·525	·584	·347	·317	55·5	G
·525	·534	·343	·316	55·4	G
·525	·493	·312	·332	67·1	F

p_H 7·5; Total Ca = 11·24 mg. %; Ca in 5 cc. serum = 0·562 mg.; CO_2 tension = 50·16 mm. Hg.

·562	·503	·312	·337	64·4	F
·562	·516	·343	·413	61·4	G
·562	·462	·462	·383	70·6	H

Dog II. p_H 7·5; Total Ca = 10·5 mg. %; Ca in 5 cc. serum = 0·525 mg.; CO_2 tension = 50·92 mm. Hg.

·525	·530	·344	·344	65·5	G
·525	·515	·367	·354	64·9	H

Dog III. p_H 7·45; Total Ca = 11·7 mg. %; Ca in 5 cc. serum = 0·585 mg.; CO_2 tension = 50·92 mm. Hg.

·585	·505	·312	·342	63·5	C
·585	·558	·343	·362	65·1	G
·585	·550	·367	·382	67·9	H
·585	·557	·367	·377	66·1	H

Dog IV. p_H 7·45; Total Ca = 11·30 mg. %; Ca in 5 cc. serum = 0·565 mg.; CO_2 tension = 44·14 mm. Hg.

·565	·480	·312	·343	66·1	F
·565	·491	·343	·323	59·1	G
·565	·511	·367	·362	63·1	H
565	·537	·367	·372	66·7	H

Dog V. p_H 7·4; Total Ca = 12·7 mg. %; Ca in 5 cc. serum = 0·635 mg.; CO_2 tension = 42·56 mm. Hg.

·635	·580	·212	·362	64·8	F
·635	·571	·343	·363	60·1	G
·635	·550	·367	·380	62·0	H
·635	·550	·367	·387	64·0	H

Dog VI. p_H 7·4; Total Ca = 12·6 mg. %; Ca in 5 cc. serum = 0·630 mg.; CO_2 tenion = 50·0 mm. Hg.

·630	·590	·312	·332	55·9	F
·630	·589	·343	·352	56·8	G
·630	·621	·367	·372	59·3	H
·630	·599	·367	·367	57·8	H

Dog VII. p_H 7·4; Total Ca = 9·0 mg. %; Ca in 5 cc. serum = 0·450 mg.; CO_2 tension = 49·4 mm. Hg.

·450	·470	·312	·312	69·3	F
·450	·481	·367	·342	70·4	H

PARTIALLY PARATHYROIDECTOMISED DOGS.

Table VI.

Dog I. Total Ca = 6·75 mg. %; Ca in 5 cc. serum = 0·337 mg.; CO_2 tension = 50·0 mm. Hg.

Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium	Compensation dialysing solution
Begin	End	Begin	End	%	
·337	·340	·210	·250	90·0	B
·337	·368	·257	·291	96·4	D
·337	·363	·312	·296	83·1	F
·337	·368	·367	·352	100·0	H

Dog II. Total Ca = 6·13 mg. %; Ca in 5 cc. serum = 0·306 mg.; CO_2 tension = 47·83 mm. Hg.

·306	·335	·257	·302	103·5	D
·306	·302	·257	·272	93·2	D
·306	·352	·312	·293	90·0	F
·306	·291	·406	·372	109·0	J

Dog III. Total Ca = 8·2 mg. %; Ca in 5 cc. serum = 0·410 mg.; CO_2 tension = 48·64 mm. Hg.

·410	·410	·257	·281	84·5	D
·410	·391	·367	·392	101·6	H
·410	·391	·406	·412	101·9	J

COMPLETELY PARATHYROIDECTOMISED DOGS.

Table VII.

Dog I. July 21, operation; July 22, hyperpnoea of Cheyne-Stokes type with salivation, fine tremor of muscles, tetany not severe; 70 cc. of blood drawn from femoral artery.

Total Ca = 5·0 mg. %; Ca in 5 cc. serum = 0·250 mg.; CO_2 tension = 49·4 mm. Hg.

·250	·314	·312	·280	99·2	F
·250	·246	·257	·221	90·0	D
·250	·310	·367	·312	102·8	H
·250	·291	·406	·322	95·2	J

Dog II. July 20, complete operation at noon; July 21, 2 p.m., tremors of neck and shoulder muscles, increased respiration with slight salivation; 8 p.m., marked tremors of muscles of the neck and of the hind limbs. Respirations very rapid and of Cheyne-Stokes type; 9 p.m., 75 cc. of blood drawn aseptically from the femoral artery under ether anaesthesia; 10 p.m., animal much improved, able to stand and able to drink on account of reduced respiratory rate; animal mentally alert.

Total Ca = 6·09 mg. %; Ca in 5 cc. serum = 0·305 mg.; CO_2 tension = 41·8 mm. Hg.

·305	·279	·257	·272	69·0	D
·305	·273	·312	·291	90·0	F
·305	·273	·367	·322	90·3	H

July 22, dog in marked tremor, capable of standing but legs stiff; respiration very irregular and rapid with a rate of over 200 per minute; 70 cc. of blood taken aseptically under ether; respiration rate normal under ether. Definite improvement for 36 hours after withdrawal of blood, the dog becoming quite lively and responsive. Tremors commenced within 36 hours, being felt over the shoulder-girdle; respiration rate again rapidly rising from 60 per minute to 180 per minute.

Total Ca = 5·1 mg. %; Ca in 5 cc. serum = 0·255 mg.; CO_2 tension = 44·84 mm. Hg.

·255	·287	·312	·261	82·0	F
·255	·299	·406	·322	93·4	J

Dog III. Total parathyroidectomy performed July 21; dog died in the night about 20 hours after operation, having developed hyperpnoea and slight tremor during the evening of the 21st; unable to obtain blood from veins because of clotting.

Dog IV. July 20, total parathyroidectomy; July 26, this dog has shown no signs of tetany.

Total Ca = 8·3 mg. %; Ca in 5 cc. serum = 0·415 mg.; CO_2 tension = 51·68 mm. Hg.

·415	·358	·367	·332	72·4	H
·415	·425	·257	·262	64·4	D

August 5, no signs of tetany have developed. Total calcium = 7·5 mg. %.

Dog V. July 22, total parathyroidectomy performed; dog in excellent condition. July 23, slight hyperpnoea, muscular twitching, and salivation developing. July 24, all the muscles of the abdomen, shoulders, head and neck and legs in constant and rapid twitching; dog is very thirsty but unable to drink because of the very rapid respiration rate amounting to 240 per minute; the dog is able to stand, is constantly on the move and constantly attempting to drink. July 24, 10 a.m., because of the severity of the condition 70 cc. of blood were drawn from the femoral artery; at 2 p.m. the condition had greatly improved; all tremors had gone and respirations were almost normal. July 26, noon, tetany attack developing with rapid respirations, salivation, muscle tremors and spasticity of the limbs. July 27, 9 a.m., severe tetany has developed with strong spasmodic contractions of muscles of the head and jaw, the latter causing sharp snapping of the teeth together, causing the tongue to be bitten. Respiration rate 250 per minute; salivation and thirst marked; animal lying soaked in perspiration. 11 a.m., because of the distressing condition animal bled to death.

July 24, Total Ca = 5·0 mg. %; Ca in 5 cc. serum = 0·250 mg.; CO_2 tension = 47·12 mm. Hg.

Ca in mg. in 5 cc. serum		Ca in mg. in 5 cc. dialysate		Diffusible calcium	Compensation dialysing solution
Begin	End	Begin	End	%	
·250	·313	·257	·254	100·4	D
·250	·313	·312	·282	100·8	F

July 26, Total Ca = 4·7 mg. %; Ca in 5 cc. serum = 0·235 mg.; CO_2 tension = 46·36 mm. Hg.

·235	·256	·257	·246	100·0	B
·235	·268	·312	·271	97·8	C

July 27, Total Ca = 4·7 mg. %; Ca in 5 cc. serum = 0·235 mg.; CO_2 tension = 42·56 mm. Hg.

·235	·305	·315	·227	96·6	F
·235	·332	·367	·300	99·1	H

Dog VI. July 22, total parathyroidectomy performed. July 30, usual signs of tetany; 70 cc. of blood taken from femoral artery for estimations and animal bled to death.

Total Ca = 4·6 mg. %; Ca in 5 cc. serum = 0·230 mg.; CO_2 tension = 50·16 mm. Hg.

·230	—	·312	·263	93·1	F
·230	·322	·367	·284	87·4	H

Dog VII. July 29, total parathyroidectomy performed; very definite signs of tetany developed within 48 hours.

Total Ca = 5·2 mg. %; Ca in 5 cc. serum = 0·260 mg.; CO_2 tension = 50·16 mm. Hg.

·260	·269	·257	·251	94·3	D
·260	·307	·367	·312	98·8	H

Dog VIII. Aug. 2, noon, total parathyroidectomy performed. Aug. 3, 9 a.m., marked tremor in muscles of the head and neck, shoulders, abdomen; respiration rate 220 per minute; 70 cc. of blood taken from femoral artery for estimations. Aug. 4, condition relieved temporarily by withdrawal of 70 cc. of blood. Aug. 5, 2 p.m., tremors of muscles very marked; perspiration and salivation marked; respiration rate about 200 per minute. 7 p.m., tremors of muscles as at 2 p.m.; haemorrhage from the tongue due to injury from spasmodic contraction of jaw muscles; perspiration profuse; spasticity of limbs marked; animal unable to stand; condition very severe.

Aug. 3, Total Ca = 4·6 mg. %; Ca in 5 cc. serum = 0·230 mg.; CO_2 tension = 50·16 mm. Hg.

·230	·280	·312	·241	73·5	F
·230	·290	·312	·240	73·0	F

Aug. 4, Total Ca = 6·0 mg. %; Ca in 5 cc. serum = 0·304 mg.; CO_2 tension = 51·68 mm. Hg.

·304	·310	·312	·302	97·6	F
·304	·309	·367	·283	98·5	H

Aug. 5, Total Ca = 5·4 mg. %; Ca in 5 cc. serum = 0·270 mg.; CO_2 tension = 49·56 mm. Hg.

·270	·179	—	·120	88·8	No compensation fluid used
·270	·138	—	·126	93·3	

NOTE. The percentage of calcium which is diffusible calcium is calculated as follows:

$$\text{Diffusible calcium} = \frac{\text{Ca in dialysate after dialysis} \times 2 - \text{Ca added}}{\text{Original calcium in serum}}.$$

in situ while all other similar tissue is removed is not par excellence the method that should be followed in the investigation of the internal secretion of any gland. Just as in partial depancreatisation so in partial parathyroidectomy, the gland or piece of gland should be transplanted and the activity of the graft definitely proved before it is totally removed. In the former method one can not with assurance state whether or not there has been any alteration in the normal blood and lymph supply due to operative interference. In all of these operative animals subcutaneous suture was employed, no dressings were applied and without exception excellent healing of the neck wounds was obtained.

It seemed that as far as the purpose of the investigation was concerned no practical end would be served by the method of partial parathyroidectomy: it was therefore resolved to perform the complete operation on all other animals in one stage.

From the average percentage of diffusible calcium recorded for these tetany animals namely 93·70, a percentage which is computed from the total amount of calcium in each sample of serum, it is evident that the loss of colloidal calcium is indeed a marked one.

It is true that the compensation in dialysing was not complete, but in view of the high percentages recorded and also in view of the results obtained on the final serum from dog VIII, in which no compensation was attempted, one is forced to the conclusion that in severe tetany the major part, if not all, of the colloidal calcium is freed from its protein combination. It would also seem from certain of the results that it would be possible to determine the rate of the breakdown of this calcium proteinate linkage. It appears however that the rapid loss of diffusible calcium from the blood demands a correspondingly rapid liberation of calcium from its colloidal state.

The great demand of the organism for calcium one can understand upon witnessing the severity of the condition. It seems feasible therefore to postulate that there is an attempt on the part of the organism to supply the bivalent salts necessary for the maintenance of a normal condition of irritability of the nervous system and the method adopted is that of a rapid liberation in ionic form of all the colloidal calcium in the blood. But unfortunately in virtue of its free state in the circulation the calcium is very readily lost by way of the kidneys, which organs being devoid of judgment and exercising no discrimination are not to be expected to respond to the abnormal condition by setting up any mechanism of curative adaption. Under these conditions it is evident that once the store of colloidal calcium in the blood is utilised, there must be a call upon the calcium of the tissues. That there is a muscle protein breakdown in tetany has been amply proved by several workers, more notably Paton, Findlay and Burns. It is possible therefore that there is a continual breakdown of muscle protein, which is responsible for the production of a toxic substance, and it is this steady decomposition of protein which is the mechanism whereby an attempt is made to meet the call of the nervous and other tissues for calcium.

In view of the urgent needs of the organism and probably as a result of the protein decomposition, the calcium is thrown out in that form in which it is most easily utilised.

In considering the reason for the presence of diffusible calcium in the blood in tetany the somewhat contradictory facts that both hydrochloric acid and calcium salts are effectual in the treatment of tetany open up a suggestion that the increase in the hydrogen ion concentration of the blood during acute tetany may be a means whereby more calcium can be liberated from protein combination. The lessened alkalinity of the blood would favour the dissolving of the calcium, and would thereby make immediately available for the body calcium as soon as it was broken out from its protein combination.

It must not be forgotten that the alteration in calcium metabolism is a sign of tetany, and that the causative factor is probably a body associated with the creatine of muscle protein.

Watanabe [1918, 1] has shown that the administration of guanidine induces severe acidosis, with a retention of phosphates and a decrease of calcium in the blood, and he expresses the possibility that the fundamental cause of tetany is the increased formation of guanidine nitrogen brought about by the disturbance of the function of the parathyroid glands. But he also points out elsewhere [1918, 2] that the tetany due to guanidine injection is not abolished by calcium administration. This last observation is contradictory to that of Fühner [1915] in which he states that the administration of calcium removes the effects of guanidine, an observation which tends still further to correlate these two conditions namely parathyroid and guanidine tetany. A factor in favour of the toxic theory which has been fully proved during this investigation is that the withdrawal of blood amounting to about 100 cc. and the injection of about 200–300 cc. of calcium-free saline, immediately effects an amelioration of the symptoms of tetany.

The signs of parathyroid tetany.

In all of these dogs with the single exception of No. IV definite symptoms of tetany developed. It is evident from the figures for dog IV that there was no derangement of the free and combined calcium balance, but it will be noted that there is a slight though definite loss in total calcium.

Dogs II, V, VI, VII and VIII developed signs of tetany within 36 hours and it will be seen from the notes in the tables that the attacks were with only two exceptions of a severe type. None of these animals suffering from severe tetany survived for more than five or six days. The signs noted as definitely indicating parathyroid tetany were the increased respiratory rate of from 200 to 250 per minute, with noisy expiration, often of a Cheyne-Stokes type, the profuse salivation, and nasal secretion, all of which developed within 48 hours. The characteristic muscle tremors were invariably in evidence within 24 hours as fine muscle twitchings which could be felt most clearly over the shoulder girdle. These tremors became more and more marked until

in some cases by the end of the second day after operation they had become coarse spasmodic twitchings. These were markedly in evidence in dogs V, VII and VIII, in which cases the spasmodic contractions of the temporal and masseter muscles were undoubtedly distressing, the latter causing injury to the tongue by the sudden snapping together of the teeth.

The spasticity of the limbs, on account of which the animal is forced to lie on its side, the inability to stand, the cardiac irregularities, vomiting, and at times profuse perspiration and loss of appetite were all in evidence two and at the latest three days after operation.

The mild or more chronic types of tetany described by others in which the animals lived for two or three weeks after operation have not been seen throughout this investigation; all were definite, and most were severe cases of tetany terminating fatally within one week. The severity of these attacks was always greatly ameliorated by the withdrawal of the 70 cc. of blood for the necessary examinations.

This severe condition occasioned by complete removal of the thyro-parathyroid apparatus may well account for the results recorded.

The hydrogen ion concentration and CO_2-combining power of the blood in tetany.

The p_H and the CO_2-combining power of the blood were determined in these dogs. The normal p_H was found to vary between 7·45 to 7·50 while within 24 hours after parathyroidectomy it was usually somewhat higher varying from 7·55 to 7·75 but with the rapid development of the tetany it invariably fell to about or below the normal and after the third day it varied from 7·2 to 7·35.

In accordance with the findings of others the signs of initial alkalosis were undoubtedly present. Following this slight increase in the p_H, there was an indication that with the falling p_H the alkaline reserve was encroached upon by non-volatile acids, and as a condition of acidosis is produced the alkaline reserve becomes more and more depleted, until subsequently the compensatory loss of carbon dioxide by way of the alveolar air, whereby the p_H is maintained normal, is of no avail and the p_H falls, and continues to fall until the death of the animal.

That such a process may take place is suggested by the figures for the CO_2-combining power of the blood which have been obtained. As far as the CO_2-combining power of the plasma is concerned the high figures of McCann [1918] have not been confirmed.

The graph (Fig. 3) shows the average findings for CO_2-combining power and the p_H of the blood arrived at in this laboratory. This aspect of the problem still requires further investigation, and it is hoped that the results of further research on this part at present being undertaken here will shortly be published.

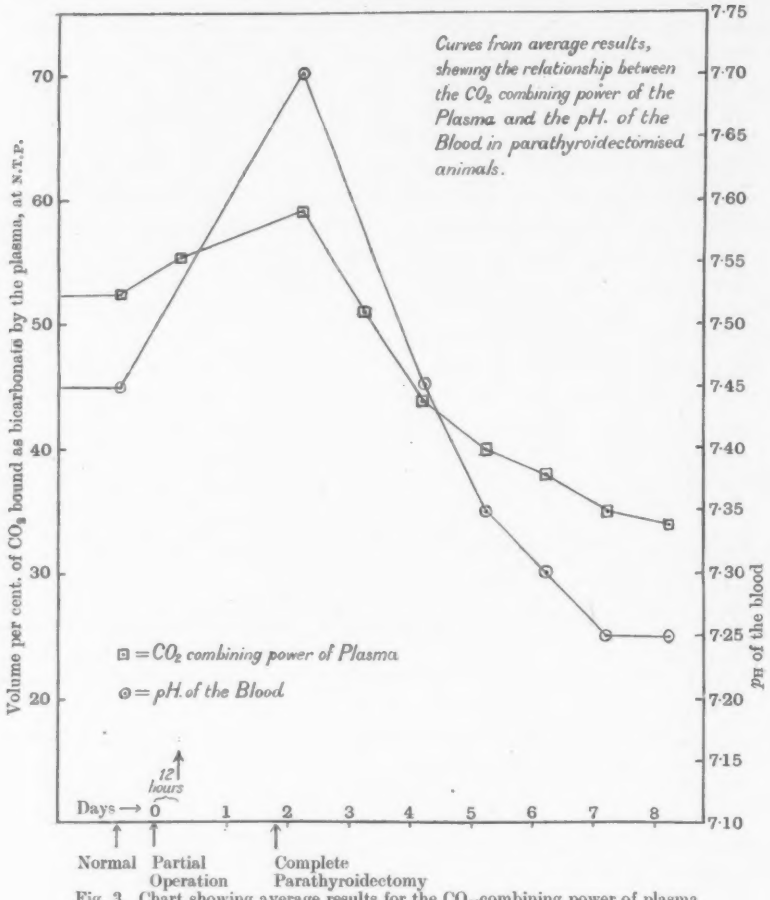

Fig. 3. Chart showing average results for the CO_2-combining power of plasma and the p_H of the blood.

Summary.

(1) The calcium content of 100 cc. of normal blood amounts to the following average figures (in mg.), total 9·12, plasma 8·11, cells 1·01; in tetany these drop to the average figures of whole blood, 5·7, plasma, 5·26 and cells, 0·46. These figures show a loss of calcium amounting to 37·2 % for whole blood, to 54·4 % for the cells and 35·2 % for the plasma.

(2) Diffusible calcium in normal serum averages from 60 to 70 %, while in severe parathyroid tetany it amounts to 94 % of the total calcium.

(3) While there is an immediate state of alkalosis following parathyroidectomy, this condition is not necessarily marked and with the development

of the signs of severe tetany it passes rapidly into a condition of acidosis as shown by a steadily falling p_H of the blood.

(4) The immediate relief of the condition consequent upon the withdrawal of 70–100 cc. of blood is indicative of a toxic causative factor.

(5) The calcium deficiency and the great loss of colloidal calcium is merely indicative of a rapid protein disintegration.

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III. THE BACTERICIDAL ACTION OF TELLURIUM DERIVATIVES OF CERTAIN ALIPHATIC β-DIKETONES.

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(*Received December 22nd, 1922.*)

In a previous paper [Morgan, Drew and Cooper, 1922] attention was drawn to the powerful inhibitory effect of the tellurium derivatives of aliphatic β-diketones upon the growth of *B. coli communis*. Attempts made to estimate the phenol coefficients of these substances in the ordinary way showed that on sub-culturing the broth tubes into fresh broth growths occurred, indicating that the bacilli had not been necessarily destroyed by the short exposures, *e.g.* 30 minutes, to solutions of the tellurium compounds. The phenol coefficients could not therefore be estimated accurately, but indirect evidence was obtained of a strong inhibitory action, and it was calculated that in the case of tellurium propionylacetone the inhibitory effect would be appreciable even in a concentration of 1 in 5,000,000.

These tellurium compounds would, therefore, be classified in the usual way as growth-inhibitors, rather than as disinfectants, but it is probably more correct to regard them as substances which possess the remarkable property of slowly exerting a marked germicidal action in extremely low concentration, and in this respect they differ fundamentally from phenols and other substances which are only active in much higher concentration.

Further experimental work has since been carried out with the following objects in view:

1. To ascertain the influence of chemical constitution on the germicidal power of these tellurium derivatives, with the object of obtaining substances of definite chemical structure fulfilling the requirements of high germicidal power, and minimum toxicity to higher animals.

2. To investigate the specific action of tellurium compounds on different micro-organisms.

For this work the usual method of determining phenol coefficients has been superseded by the following process, in which a comparative basis has been introduced as an essential condition of the inhibitory method.

Experimental method.

To constant volumes (8 cc.) of Lemco-peptone broth (acidity = + 7E) varying volumes of sterile distilled water were added, so that on the subsequent addition of requisite amounts of the tellurium β-diketone solutions, the total volume would in each case be 10 cc. Before the addition of the germicidal substance, however, one drop[1] of a 24 hours' culture of the test-organism was added to each tube. After thoroughly mixing, the disinfecting solutions were added, and after repeated mixing the tubes were incubated at 37° for 48 hours. In this way a definite number of organisms were subjected to the action of the various concentrations of germicide for a definite period in the presence of a sufficient food supply. For each experiment a series of phenol control-tubes was set up in a similar way and at the end of 48 hours the phenol coefficients could thus be calculated.

This method of experiment conforms more closely with actual conditions and is thus of more practical value than the ordinary phenol coefficient test in investigating the action of germicides of possible use for internal application, inasmuch as the disinfectant is allowed to act slowly over a considerable period at body temperature and in the presence of organic matter.

It should be pointed out that if the culture tubes were subcultured into fresh broth, no growths occurred on subsequent incubation. This result demonstrates that in these experiments the tellurium compounds were really exerting a bactericidal action, and were not only growth-inhibitors.

The phenol coefficients have previously been expressed in terms of concentrations of *grams per cc.* We consider it, however, more accurate to calculate the results in terms of molecular weights, and our results are therefore set forth as equimolecular phenol coefficients, *i.e.*

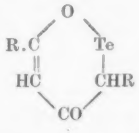
$$\text{phenol coefficients} \times \frac{\text{molecular weight of substance tested}}{\text{molecular weight of phenol}}.$$

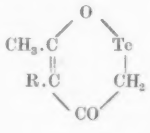
In certain cases considerably different results are obtained.

The β-diketones employed in producing the tellurium compounds are acetylacetone and its homologues with the substituted alkyl group attached either to a terminal carbon atom or to the central one (position 3) as shown by the following formulae:

$$R \cdot CH_2 \cdot CO \cdot CH_2 \cdot CO \cdot CH_2R, \quad CH_3 \cdot CO \cdot CHR \cdot CO \cdot CH_3.$$

The general structures of the corresponding types of tellurium derivatives are to be represented as follows:





[1] We have employed smaller and also larger numbers of organisms in different experiments, but the results are not appreciably affected by variation in numbers.

For convenience of terminology these substances are referred to in the following table as derivatives of methane.

The organism employed in the experiments was *B. coli communis*, and it was found that while the resistance to phenol was remarkably constant, that of the same organism to the tellurium compounds varied considerably. For this reason the results in column II in the following table are expressed as ranges of concentration through which the substances were found to be bactericidal, while in column III a characteristic mean concentration is set forth. For convenience the concentrations are expressed in grams per cc. but in column IV the phenol coefficients are "equimolecular" coefficients and represent accurately the relative germicidal powers of the substances examined.

Table I.

	Substance	Range of minimum concentration bactericidal to *B. coli*	Mean bactericidal concentrations	"Equimolecular" phenol coefficients
1.	Tellurium diacetylmethane	1 in 270,000– 750,000 (4)[1]	1 in 500,000	2,800
2.	,, 3-methyldiacetylmethane	1 in 550,000– 1,000,000 (4)	1 in 900,000	4,000
3.	,, 3-ethyldiacetylmethane	1 in 1,250,000– 3,500,000 (6)	1 in 2,500,000	13,000
4.	,, acetylpropionylmethane	1 in 1,000,000– 5,500,000 (5)	1 in 3,000,000	14,000
5.	,, dipropionylmethane	1 in 7,000,000–10,000,000 (6)	1 in 9,000,000	45,000
6.	,, acetylbutyrylmethane	1 in 1,000,000– 5,500,000 (10)	1 in 3,000,000	15,000
7.	,, propionylbutyrylmethane	1 in 1,500,000– 4,500,000 (4)	1 in 2,800,000	14,000
8.	Phenol	1 in 510– 590	1 in 550	—

[1] The figures in brackets indicate the number of tests.

A consideration of the tabulated results shows that these tellurium compounds possess an extraordinarily high bactericidal power. The equimolecular phenol coefficients for the different members of the series range from 2,800 to 45,000 even in the presence of organic matter, the most active member being tellurium dipropionylmethane, which is bactericidal in a dilution of 1 in 9,000,000.

The bactericidal action increases considerably as the homologous series is ascended, until with a third substitution of the methyl group (as in tellurium propionylbutyrylmethane) a limit is reached and there is even a diminution in germicidal activity.

The efficacy of the compounds is furthermore influenced by the chemical structure of the β-diketone, position isomerism playing a very important part in determining bactericidal power. Thus, tellurium acetylpropionylmethane and tellurium 3-methyldiacetylmethane are isomeric, the methyl group in the former being attached to a terminal carbon atom, in the latter to the central atom, in position 3. Tellurium dipropionylmethane and tellurium 3-ethyldiacetylmethane form a similar pair of isomerides. In each case it is seen that a terminal substitution of methyl group enhances bactericidal power to a very much greater extent than when substitution is effected centrally.

Again, tellurium dipropionylmethane, tellurium acetylbutyrylmethane and

tellurium 3-ethyldiacetylmethane are all three isomeric, the chemical constitution of the three β-diketones from which they are derived being respectively

(1) $CH_3 . CH_2 . CO . CH_2 . CO . CH_2 . CH_3$,

(2) $CH_3 . CH_2 . CH_2 . CO . CH_2 . CO . CH_3$,

(3) $CH_3 . CO . CH(C_2H_5) . CO . CH_3$.

The results set forth in Table I show that the tellurium compound of the symmetrical β-diketone (1) is much more efficacious than that of the dissymmetrical isomeride (2), which in turn is somewhat more active than the third isomeride, where substitution has been effected entirely in the central position.

Results of much the same order have been obtained with a different strain of *B. coli*.

A few experiments with the following organisms have also been carried out: (1) *B. paratyphosus A* and *B*; *B. typhosus*; (2) *Streptococcus haemolyticus*, *Staphylococcus pyrogenes aureus*, and the results so far indicate that the foregoing tellurium compounds exert a selective action on coliform organisms, the *cocci* on the other hand being much more resistant. In this respect the substances resemble benzoquinone [Cooper, 1912; Morgan and Cooper, 1921] and telluric acid [Joachimoglu, 1922] which has been found by the latter to be selectively inhibitory to coliform organisms in a concentration of 1 in 40,000.

The authors are extending the investigations to other pathogenic organisms including the acid-fast group, and, with the object of ascertaining whether these tellurium compounds can be employed for the purpose of inner disinfection, experiments are shortly being carried out to determine their toxicity to higher animals.

The authors desire to express their thanks to the Advisory Council of the Department of Scientific and Industrial Research for grants which have helped to defray the expenses of this investigation.

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IV. CALCIUM CONTENT OF THE BLOOD DURING PREGNANCY.

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(*Received December 22nd, 1922.*)

The importance of calcium in growth is well established, and in the human foetus it has been shown that calcium retention varies very greatly at the different stages of development.

Paul Bar [1907] by estimating the amount of calcium found in foetuses of different ages concluded that the absorption varied considerably with the stage of growth and was of the order shown in the following table (calcium expressed as calcium oxide):

0·0048 g.	each day	up to	120th day.	
0·083	,,	,, from	120th–150th	day.
0·087	,,	,, ,,	150th–180th	,,
0·084	,,	,, ,,	180th–210th	,,
0·638	,,	,, ,,	210th–270th	,,

From these figures it would appear that at certain periods there is an abrupt alteration in the calcium retention which must mean a sudden change in the demands upon the maternal stores of that element.

The question then arises does the blood of the mother show at these stages an alteration in its calcium content (an increase if the blood carries more calcium to meet the heavier demand made upon it, or a decrease if the demand is not counterbalanced by a greater mobility of the calcium), or is the calcium metabolism so perfectly balanced that the blood maintains a constant value in spite of the varying demands made upon it? In an attempt to answer this question, a number of analyses of the blood of women at different stages of pregnancy have been made, paying particular attention to those periods at which the abrupt changes in the calcium metabolism of the foetus seem to occur. The samples were taken 3rd–4th month, 5th month, 6th–7th month, and 8th–9th month.

Method.

The analysis was always made on serum, and not on the whole blood. The calcium is present in very different amounts in the corpuscles and plasma, and unless therefore the proportion of corpuscles to plasma is ascertained, the true value of the calcium content cannot be arrived at. Some of Lyman's varying results may be due to his always making use of whole blood. The method finally adopted was based on that of McCrudden [1912] modified for small quantities.

The working out of this method was started in 1919, and it was found that a similar method was being adopted by other workers for estimating small quantities of calcium. 5 cc. of serum were found to be sufficient and concordant analyses could be made when this amount was available.

Precipitation of the protein.

The protein of the serum is precipitated with 6·5 % trichloroacetic acid, the volume taken being five times that of the serum, a rather larger ratio than that used by Lyman [1917, 1, 2]. The serum is added very slowly drop by drop from a pipette to the trichloroacetic solution in a small flask, rotating the liquid continuously. The flask is then stoppered and the contents thoroughly mixed by shaking. After standing for about half-an-hour, the mixture is filtered through a very small calcium-free filter paper, and an aliquot part of the filtrate is taken for the estimation of the calcium present.

Precipitation and estimation of the calcium.

The aliquot part of the filtrate is made just alkaline with concentrated ammonia, added drop by drop, using methyl red as indicator, then just acid with $N/2$ hydrochloric acid, added drop by drop from a burette or 1 cc. pipette. To this is added 1 cc. of $N/2$ hydrochloric acid, 1 cc. of oxalic acid solution 4 %, and 1 cc. of a 20 % sodium acetate solution, stirring or shaking carefully while adding the latter. The solution is allowed to stand for a day, and the precipitate of calcium oxalate is separated by means of a high speed centrifuge and washed twice with water, centrifuging between each washing. The calcium oxalate so obtained is dissolved in 4 cc. of N sulphuric acid made just pink with $N/100$ potassium permanganate, heated to about 70° on a water-bath, and then titrated with $N/100$ potassium permanganate solution, made according to the direction of Halverson and Bergeim [1917]. In this estimation of course all materials used must be tested for calcium, and only calcium-free materials employed. The water is always twice distilled and every care must be taken throughout the estimation to protect the solutions from contamination with dust or other reducing matter. Kramer and Howland [1920] described a method for estimating calcium in 1 cc. of serum, and on testing this method satisfactory results were obtained. This method was therefore occasionally adopted when only 2 cc. of serum were available, duplicate estimations always being made.

Results.

The first series of analyses were made during 1920 to 1921, and in these there was no attempt to follow the same case throughout the different stages. The figures for this series, given below in Tables I and II are therefore the mean of experiments made on different women at the same period of pregnancy. The specimens of blood were obtained from maternity patients attending at the Royal Free Hospital.

The amount of calcium in the following tables is always given in milligrams of calcium per 10 cc. of serum.

Table I. (1st Series 1920–21.)

Stage month	Number of specimens analysed	Average calcium found	Range of calcium found	
			Highest	Lowest
3–4	12	0·968	1·17	0·763
5–5½	10	1·00	1·29	0·72
6–7	29	1·02	1·2	0·72
8–9	15	0·966	1·25	0·74

The above table shows little variation between the highest and lowest amounts of calcium in 10 cc. of serum at the different stages. Usually the range is from 1·05 to 0·93. These results seem to bear out De Wesselow's conclusion [1922], that during the last months of pregnancy there is a tendency to a fall in the calcium content of the serum. The results above are taken from all the cases examined during the year 1920–21 without separating the normal cases of pregnancy from those who in previous pregnancies had had miscarriages or still-born infants. In Table II this separation has been made.

Table II.

Stage month	Number of specimens analysed	Average calcium found normal	Numbers of specimens analysed	Average calcium found in cases previously abnormal
3–4	10	0·97	2	0·96
5	4	1·13	6	0·916
6–7	25	1·024	4	0·989
8–9	11	0·957	4	0·992

The numbers in Table II show that in the normal cases there is a decided drop in the calcium content during the last month of pregnancy, while in the other cases the difference in the calcium content at the different stages is not so marked, the calcium being slightly higher in the last month than at the beginning. The number of these latter cases is small, and a larger and more complete set is being investigated.

From the wide range of values for any given month in different cases it was clearly seen that for a definite answer to the problem the analyses must be made on the *same* individual at the different periods. A second series of analyses on these lines was carried out during the year 1921–22. In this way compensation was made for any individual idiosyncracies causing a variation

in results. In the second series in as many cases as possible analyses of the blood calcium have also been made within 14 days after confinement to determine the effect of lactation on the calcium content. Analyses are also now being made of the blood calcium of cases 2–3 months after confinement to investigate the effect of continued lactation. These results are given in Table III.

Table III. (2nd Series 1921–22.)

Stage Month	Number of specimens analysed	Average calcium found	Range of calcium found	
			Highest	Lowest
3–4	22	1·11	1·28	0·898
6–7	24	0·996	1·17	0·848
8–9	24	0·94	1·03	0·82
Post-confinement within 14 days	18	0·994	1·15	0·814

These results are in accord with those of last year and show a decrease in the calcium content of the serum during the last month of pregnancy, and a tendency to rise after confinement and during the early stages of lactation.

As stated earlier in this paper, during this year an endeavour was made to obtain the calcium content of the serum of the *same* woman at the above different stages of pregnancy, and this has been successfully carried out in a number of cases. In some, however, the series are not complete owing to the women failing to come to the hospital at one or other of the periods under investigation. The more complete figures are given in Table IV.

Table IV.

Case	Month 3–4	Month 6–7	Month 8–9	Post-confinement
4	1·23	1·1	—	1·07 (10 days)
7	1·09	0·916	0·775	1·07 (10 days)
8	0·898	0·93	0·92	—
9	—	1·17	1·00	—
12	1·17	0·88	—	1·05 (5 days)
13	0·913	0·898	0·92	1·01 (6 days)
14	1·2	0·948	—	1·04 (10 days)
16	—	0·944	0·96	0·814 (13 days)
17	—	1·15	0·866	—
18	—	0·97	0·82	0·999
20	1·17	0·943	0·91	0·999 (7 days)
22	—	1·03	0·93	1·06 (13 days)
23	1·17	1·11	0·983	—
29	—	0·85	1·03	—
30	—	0·943	0·897	0·889
36	—	0·91	—	0·999 (13 days)
38	—	0·95	—	1·02 (7 days)
41	0·93	1·07	0·88	—
42	—	—	0·984	1·15 (14 days)
44	—	0·856	0·92	—
45	—	—	0·79	0·933 (10 days)

From a study of these numbers it is seen in almost all the cases there is tendency to a decrease of calcium content of the blood toward the last months of pregnancy and a general tendency to rise directly after confinement. In

cases 13, 16, 29, 44 the results do not seem to be in accord with this general tendency, but in 13 and 16, the calcium content of the serum throughout pregnancy varies very little. These variations may be due to some other concurrent condition of the system at the time of observation. In one case of nephritis recently investigated the calcium content of the serum was found as low as 0·32 mg. per 10 cc.

Other workers in this field do not seem to have observed the above regular rise and fall in the calcium content of the blood at the different stages of pregnancy. Jansen [1918, 1, 2] thinks that the calcium content is little altered by pregnancy. He worked however on the whole blood and the disadvantages of this have been already indicated. Lamers [1912] thinks that the calcium content in blood in pregnant women is higher than that of non-pregnant women. Blair Bell [1908] showed in experiments on hens that the calcium content of the blood always rose previous to the laying of an egg and then immediately fell, there being a continuous rise and fall in the case of a hen that laid an egg on alternate days. According to this the calcium content of the blood of a woman ought to rise immediately before the birth of the infant and drop immediately after. The estimates given in the previous tables have not been made later than during the 8th and 9th month—never the day before the confinement. Also the earliest time after confinement that specimens of blood have been obtainable is five days. It would be interesting to examine the blood the day before and the day after confinement in the light of Bell's results.

The fact that the foetus is constantly taking in larger and larger quantities of calcium during growth, especially large quantities during the last two months of pregnancy, and that the maternal blood shows a distinct tendency for its calcium content to decrease during the 8th and 9th months of pregnancy indicates the importance of a sufficiency of calcium in the diet of the mother during this time. Otherwise the calcium needs of the foetus may be supplied by withdrawing too large an amount from the tissues of the mother (*e.g.* bone, teeth), as it is evident from the figures in the above tables that the blood tends to maintain a fairly constant value in spite of the heavy demands made upon it. The serum of the blood of a certain number of cases has been examined for calcium where there have been continuous miscarriages or still-born infants, and where a variation might be expected. So far no very definite relationship seems to be shown. These results are given in Table V.

Number 14 gave a calcium content on one occasion a week after miscarriage of 1·1 mg.

The case of a haemophylliac was interesting. The calcium content of the serum per 10 cc. at the 7th month of pregnancy was 0·66 mg., at the 8th month 1·05 mg., and after being treated with calcium chloride for a month, at the 9th month was 1·12 mg. About nine months later the same case when not pregnant gave 0·83 mg. of calcium per 10 cc. of serum, and after treating with calcium chloride gave 0·94 mg. of calcium.

Table V.

Case	Characteristics of case	Calcium found	Percentage increase or decrease of calcium compared with average. (Calcium average 0·99 mg. per 10 cc. of serum.)
1	Sterile	1·14	15·1 +
2	Five miscarriages	1·23	24·2 +
3	Still birth	1·09	10·1 +
4	Three miscarriages	1·08	9·09 +
5	Still birth	1·03	4·04 +
6	Twelve miscarriages	1·00	1·01 +
7	Six still-born	1·00	1·01 +
8	Several miscarriages	0·961	2·92 −
9	Several miscarriages	1·02	3·03 +
10	Several miscarriages	0·98	1·01 −
11	Four miscarriages and one still-born	0·996	0·6 −
12	Six miscarriages	0·918	7·2 −
13	Four premature births	0·88	11·11 −
14	Four miscarriages	0·74	25·2 −
15	Several miscarriages	0·561	43·3 −

Investigations have also been made into the question of the variation of the calcium content of the blood in connection with menstruation. These results have already been published [Widdows, 1922]. Since the calcium content of the blood of pregnant women is at the commencement of pregnancy slightly higher than that of non-pregnant women it was expected that there might be a rise in the calcium content at the beginning of menstruation. Bell [1908] had indicated that there was a rise just before menstruation followed by a very marked drop. Sherman, Gillett and Pope [1918] could show no distinct monthly cycle in the metabolism of nitrogen, phosphorus or calcium in the case of women. Although in two of the cases referred to in the *Lancet* of November 11th, 1922, there was a suggestion of a rhythmic rise and fall in the calcium content of the serum of the blood, the variations shown by the figures as a whole are not greater than those obtained in any normal series of estimations of blood calcium, and do not support the idea of a cyclic variation in the calcium content of the blood. The question of the relationship of the amount of calcium to the phosphorus in the blood has always been kept in view, and it was intended to take up that point this coming year, but de Wesselow [1922] has already examined his cases for this relationship and shows that the amount of phosphorus decreases in the later phases of pregnancy similarly to the calcium, but that during lactation there is a relatively high value for phosphorus in the plasma compared with that of the non-lactating female.

This work emphasises the perfect regulation of the human organism to the many and varied demands made upon it. The very great demand that undoubtedly at certain stages of its development is made by the foetus for calcium, is certainly met by a very perfectly balanced calcium metabolism, as at no time is the variation of the calcium content of the serum very marked. Even so in the later stages of pregnancy there does seem to be a distinct tendency for the calcium content of the blood to decrease. This indicates that at this time attention should be paid to the diet of the mother from the point

of view of its calcium content. During the last two months of pregnancy milk (on account of its high percentage of calcium) should be given the mother in a larger amount than that included in the average adult's diet and should be considered a necessary constituent of her diet.

I have to thank the Medical Research Council for the help that I have received during the present investigation.

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V. A METHOD FOR THE QUANTITATIVE ESTIMATION OF CHOLINE IN BLOOD.

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(*Received January 1st, 1923.*)

The chemical methods at present available for the quantitative determination of choline are unsatisfactory. Those most commonly employed depend on the compounds formed with the heavy metals. But these are fairly soluble in water, in some cases to the extent of 25 %. The aurichloride is the least soluble, one part dissolving in 75 parts of water.

The biological method of testing the acetylated product upon the frog's heart suggested by Reid Hunt [1915] and later by Führer [1916] can at present be regarded as affording only a rough estimation of the amount of choline.

The method of detecting the presence of choline in blood and cerebro-spinal fluid described by Allen [1904] seemed to promise the possibility of being developed into a quantitative process. Staněk [1905] described such a process in which he either estimated the nitrogen of the choline compound, or decomposed the periodide with cuprous chloride. The details of the second method were criticised by Kiesels.

The following method has been devised and tested, and it appears to be free of the difficulties of Staněk's procedure.

The principle is to extract the choline from the blood or tissues and to precipitate it with iodine as periodide, to wash off any free iodine and to decompose the periodide with dilute nitric acid, finally to extract the free iodine with chloroform and estimate it using a standard solution of sodium thiosulphate.

1 cc. *N*/20 thiosulphate sol. = 0·0007 g. choline HCl.

From 20 to 50 cc. of blood are drawn off into about four times this volume of absolute alcohol and well shaken. The containing vessel is allowed to stand for about 12 hours, after which time the contents are filtered into an evaporating basin. The precipitate is rubbed up and well washed with absolute alcohol and then is added to the filtrate. The filtrate is evaporated to a syrup and taken up in distilled water. The possibility of the extraction and decomposition of lecithin with the liberation of choline at this stage is considered later. The fluid is again evaporated to a syrup and the process repeated till it is alcohol-free. The solution is then transferred to a dialysing thimble of about 15 cc. capacity and dialysed for 24 hours into 200 cc. distilled water.

It is advisable to repeat this dialysis and to combine the resulting liquids. This is then evaporated to about 5 cc. and carefully washed into a beaker keeping the bulk as low as possible. 20 to 30 vols. of saturated iodine in potassium iodide solution are now added and the beaker set aside over night to allow the choline periodide to crystallise. The crystals are then filtered through a Gooch crucible on a layer of fine asbestos wool. They are washed repeatedly with ice-cold water until free from the precipitating solution. The Buchner flask is cleared out with cold water. The crucible is placed in position for filtering and the choline periodide is now ready for decomposition. This is accomplished by a mixture of 2 parts diluted nitric acid (1 part HNO_3 to 1 part water) with 1 part chloroform.

The above mixture is then shaken and added to the crucible. The periodide is decomposed by the nitric acid and the free iodine is taken up by the chloroform and carried into the flask by the suction. This is repeated several times until the asbestos is quite white. The crucible is now removed, more chloroform is added to the flask and well shaken in order to extract any iodine dissolved in the water.

The chloroform layer is then washed acid-free by decantation with repeated quantities of cold water.

The liberated iodine in the chloroform layer is now most conveniently titrated by $N/20$ sol. of sodium thiosulphate until the pink colour just disappears.

Choline hydrochloride added to normal blood:

A. Blood, 20 cc.; choline HCl, 4·9 mg.; cc. $N/20$ thiosulphate, 6·4; recovery = 6·4 × 0·0007 = 4·48 mg. = 91·5 %.

B. Blood, 20 cc.; choline HCl, 1·9 mg.; cc. $N/20$ thiosulphate, 2·55; recovery = 94 %.

C. Blood, 20 cc.; choline HCl, 17·0 mg.; cc. $N/20$ thiosulphate, 22·0; recovery = 90·6 %.

Tests on normal blood without the addition of choline:

Blood, 25 cc.; no choline HCl.

No precipitate of periodide was produced even on long standing.

The possibility of lecithin breaking down is small if the process is carried out with reasonable speed. Tests performed on blood to which no choline was added gave negative results. If there was any free choline present its concentration was below the limit of recovery by this process.

My thanks are due to Professor Noël Paton for his help and advice.

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VI. THE INORGANIC PHOSPHORUS CONTENT OF THE BLOOD OF NORMAL CHILDREN

PRELIMINARY COMMUNICATION

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(*Received January 1st, 1923.*)

Attention has recently been directed by Howland and Kramer [1921] to the fact that the inorganic phosphorus content of the serum is lower in rachitic than in normal children. Hess has confirmed this finding [Hess and Unger, 1922], but states [Hess and Lundagen, 1922] that in some cases in which the presence of rickets was undoubted he found no reduction. He has further called attention to the fact that a slight seasonal variation of the amount of inorganic phosphorus in the blood of healthy children occurs, the highest figures being obtained in July, and the lowest in March. György [1922] and von Meysenbug [1922] have also published figures showing the reduction of inorganic phosphorus in the blood of rachitic infants. Furthermore, considerable attention is at present directed towards the question of the possibility of producing rickets in rats by a deficiency of phosphoric acid in the diet. It seemed desirable to repeat and extend these observations, and the prevalence of rickets in the slum districts of Glasgow from which our hospital patients are drawn has been taken advantage of to prosecute such a study. This investigation is still in progress.

One of the first essentials in undertaking an investigation like the present one is, of course, a knowledge of the amounts of inorganic phosphorus to be found, and the range of physiological variation occurring in the blood of healthy children. Table I shows the results of other investigators.

Table I.

Authors	Inorganic phosphorus as mg. per 100 cc.		
	Whole blood	Plasma	Serum
Howland and Kramer [1921]	—	—	4·5 to 6·8
Tisdall [1922]	—	—	4·6 to 6·4
György [1922]	5·2	—	—
Hess and Unger [1922]	4·0 to 4·8	—	—
Von Meysenbug [1922]...	—	—	4·1 to 5·8
McKelleps, De Young and Bloor [1921] ...	—	1·2 to 4·4	—

The results agree fairly closely and indicate that the range of variation, if one excludes the very low figure of 1·2 given by McKelleps, De Young and Bloor, is 4·0 to 6·8 mg. per 100 cc.

In the course of a series of estimations of the phosphorus contents of the blood and serum for comparison with rachitic figures, one or two interesting points emerged and are therefore recorded in this preliminary report.

Experimental error.

The method used throughout these experiments is that of Bell and Doisey [1920]. Marriott and Haessler's technique [1917], as well as that of Tisdall [1922] and that of Bloor [1918] take much longer to complete without giving more accurate results.

Myers and Shevky [1921] have recently criticised the method of Bell and Doisey and state that more accurate results are obtained when the standard contains not over 0·25 % more phosphorus than the specimen to be examined. They further observed that blood sometimes requires more of the molybdic acid and quinol solution than does serum. For this reason the standards used throughout this investigation are as far as possible made up to contain a quantity of phosphorus similar to that of the blood sample to be tested, and for whole blood double the quantities of molybdic acid and quinol advocated by Bell and Doisey are used.

In order to gain an estimate of the experimental error involved in the use of the method 14 consecutive estimations on the same blood sample were carried out. In each case 5 or 10 cc. of lysed diluted dog's blood were separately treated and subjected to colorimetric comparison with a freshly made up standard. The calculated values of phosphorus per 100 cc. of the unknown are given in Table II.

Table II.

	Quantity of diluted blood used cc.	Estimated values of P per 100 cc. of diluted blood mg.
1	10	1·7
2	10	1·9
3	10	2·0
4	5	1·8
5	10	2·1
6	10	2·0
7	5	1·8
8	5	1·8
9	10	2·0
10	10	1·9
11	10	1·9
12	5	1·9
13	10	2·1
14	10	1·7
		Average 1·9 $^{+0·2}_{-0·2}$

From the series of figures it is estimated that the error is plus or minus 0·2 when the average is 1·9, or roughly 10 %. This is therefore taken as the margin of experimental error.

THE DISTRIBUTION OF INORGANIC PHOSPHORUS IN THE BLOOD.

An interpretation of the published results of inorganic phosphorus estimations in normal children is, as in the case of calcium, rendered somewhat difficult in view of the fact that in some cases serum, in others plasma, and in still others whole blood has been used, since our knowledge of the distribution of the inorganic salts is at best very incomplete.

Howland and Kramer [1921] pointed out that a definite increase in the phosphorus of the serum results if the latter is allowed to stand in contact with the clot at room temperature for any length of time. This introduces a grave source of error in serum estimations and renders any investigation into the distribution of the inorganic phosphorus between corpuscles and plasma particularly difficult. The error which may result from this change is illustrated by two different series of blood and serum estimations in normal children. In every case the whole blood was immediately shaken up in 15 cc. of distilled water, 5 cc. of trichloroacetic acid added and the flask vigorously shaken and allowed to stand ten minutes before filtering off the protein. In the first series of cases no time limit was set for the separation of the serum from the clot though all were ready for filtration within three hours of the collection of the blood. The blood samples of the second series were subjected to 40 minutes' centrifugalisation immediately after venipuncture and the serum was then quickly poured off. The results are collected in Tables III and IV.

Table III. Series 1.

Blood P estimated immediately and serum P estimated after standing from 1½ to 3 hours in contact with the clot.

Number	Age (years)	Sex	Inorganic P of whole blood mg. per 100 cc.	Inorganic P of serum mg. per 100 cc.
1	8	Male	5·1	6·1
2	8	,,	5·4	7·2
3	7	,,	—	6·0
4	9	,,	5·1	5·5
5	10	,,	4·8	6·0
6	8	,,	4·0	4·8
7	12	,,	—	6·8
8	13	,,	4·8	6·5
			Average 4·86	Average 6·1
			Range of variation 4·0 to 5·4	Range of variation 4·8 to 7·2

Table IV. Series 2.

Inorganic P of whole blood estimated immediately. Serum in every case separated from clot within 40 minutes of venipuncture.

Number	Age (years)	Sex	Inorganic P of whole blood	Inorganic P of serum
1	3	Female	—	4·6
2	5	Male	4·2	4·2
3	6	,,	4·0	4·2
4	6	,,	4·8	5·1
5	7	,,	4·2	4·4
6	9	,,	5·1	5·4
7	10	,,	4·8	4·7
8	11	,,	5·1	5·1
			Average 4·6	Average 4·7
			Range of variation 4·0 to 5·1	Range of variation 4·2 to 5·4

It is quite evident that all the serum results of Series 1 are higher than those of Series 2 and since the estimations were carried out within a few days of each other and the same standard solutions employed, one can only conclude that the difference must be ascribed to the greater length of time taken to separate the serum in the first series.

The fact that the serum phosphorus is so variable, therefore, seems to render it unsuitable for comparative estimations, particularly since we know little of the changes which may be taking place during the 30 minutes which must of necessity elapse before any quantity of serum can be separated off.

Denis and von Meysenbug [1922] have criticised the use of plasma on the ground that they found citrate and oxalate to be a source of considerable error in the use of the Bell-Doisey method.

From a consideration of these points it appears that whole blood is most suitable for use in estimating the inorganic phosphorus, for though a definite increase also occurs in the lysed blood on standing each specimen can be subjected to exactly the same procedure immediately it is withdrawn from the vein.

A study of the figures given in Table IV leaves no doubt that the whole blood if used at once gives exactly the same results as serum which has been separated from the clot within 40 minutes. This leads one to suppose that the corpuscles contain exactly the same amount of inorganic phosphorus as the serum, or that, if the corpuscles are richer in this element than the serum or plasms, as suggested by Bloor [1918], a very rapid change in the distribution of the blood phosphates must take place during the process of clotting. As a standard for comparison with the rachitic figures the collected results for the whole blood of all the normal children available are grouped in Table V.

Table V.

Number	Date 1922	Sex	Age	State of health and nutrition	Inorganic P of whole blood in mg. per 100 cc.
1	July	Male	9 mths.	Normal	5·2
2	,,	Female	5 yrs.	,,	4·2
3	,,	Male	6 ,,	,,	4·0
4	,,	,,	6 ,,	,,	4·8
5	,,	,,	7 ,,	,,	4·2
6	,,	,,	8 ,,	,,	5·1
7	,,	,,	8 ,,	,,	4·0
8	Aug.	,,	9 mths.	,,	6·6
9	,,	,,	9 yrs.	,,	5·1
10	,,	,,	9 ,,	,,	5·1
11	,,	,,	10 ,,	,,	4·8
12	,,	,,	10 ,,	,,	4·8
13	,,	,,	11 ,,	,,	5·1
14	,,	,,	12 ,,	,,	5·3
15	,,	,,	13 ,,	,,	4·8
16	Oct.	,,	15 mths.	,,	4·1
17	,,	,,	14 ,,	,,	4·8
18	Nov.	,,	8 ,,	,,	5·0
19	,,	,,	8 ,,	,,	6·6
20	,,	,,	14 ,,	,,	6·2
21	Dec.	,,	3 ,,	,,	5·5
22	,,	Female	2½ yrs.	(Convalescent pneumonia)	4·0
23	,,	Male	3 mths.	Normal	4·7

Average = 4·9

Range of variation 4·0 to 6·6

The results in Table V show that the average normal phosphorus content of the blood in children is 4·9 mg. per 100 cc. The range of variation allowed for by a probable experimental error of 10 % is 4·4 to 5·4, whereas the actual range is 4·0 to 6·6. The variation in the normal amount of inorganic phosphorus in the blood is therefore not entirely due to experimental error and no figures between 4·0 and 6·6 mg. per 100 cc. can be regarded as abnormal. Children under two years undoubtedly give slightly higher results than those over three years, while in two adults whose blood has been examined the results are still lower than those obtained from the older children. That is to say, the inorganic phosphorus of the blood appears to fall with increase in age, as pointed out by Howland and Kramer, but the seasonal variation described by Hess and Lundagen [1922] has not been apparent from the figures so far available.

Effect of feeding on the normal blood phosphorus content.

In the case of the younger children some were breast fed and some bottle fed on different diets, but there appeared to be no connection between the type of feeding and the phosphorus values obtained. The question as to whether an increase in the inorganic phosphorus may result from the recent consumption of phosphorus-rich diet is, however, one which must be raised, since it seems not improbable that the slight variations obtained may be due to differences in the stage of digestion. For the purpose of throwing light on this problem the following experiment is recorded. In the course of a parathyroidectomy investigation carried out by Professor Noël Paton and Mr Watson, whom I have to thank for permission to publish this result, the latter took from the jugular vein of a dog samples of blood, (*a*) after a period of fasting, and (*b*) at increasing intervals after the ingestion of a quantity of lean meat.

The results of the experiment are tabulated below.

Table VI.

	Time	Remarks	Inorganic P of whole blood in mg. per 100 cc.
Exp. 1	9.30 a.m.	After 15 hours' fasting	2·8 %
	11.30 a.m.	„ 17 „ „	2·9
	2.30 p.m.	„ 20 „ „	3·0
	6·30 p.m.	„ 24 „ „	2·8
Exp. 2	1 p.m.	No food for 6 hours. Given 200 g. lean meat	—
	2 p.m.	1 hour after food	2·97
	3 p.m.	2 hrs. „ „	3·17
	4 p.m.	3 hours „ „	2·97

The above figures show that the amount of inorganic phosphorus in the blood is uninfluenced by fasting for 24 hours and by the recent ingestion of a quantity of food rich in phosphorus.

Conclusions.

1. The range of experimental error in the Bell-Doisey method of estimating the inorganic phosphorus of the whole blood was found to be not more than plus or minus 10 %.

2. The inorganic P content of the whole blood of 24 normal children between 3 months and 13 years of age ranges between 4·0 and 6·6 mg. per 100 cc. Average 4·9.

3. Blood and serum give the same results provided the blood protein is precipitated immediately and the serum is separated from the clot within 40 minutes.

4. The use of serum which has been allowed to stand in contact with the clot for longer periods may be a source of considerable error. It is therefore better to use the whole blood.

5. Twenty-four hours' fasting and the recent ingestion of a diet rich in phosphorus failed to produce any demonstrable variation in the inorganic blood phosphorus content of a normal dog.

I desire to express my thanks for advice and criticism to Professor Noël Paton and Dr Leonard Findlay, under whose direction this investigation was carried out. All expenses connected with the work have been defrayed by the Medical Research Council.

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VII. SEASONAL VARIATIONS IN THE DISSOLVED OXYGEN CONTENT OF THE WATER OF THE THAMES ESTUARY.

WITH SPECIAL REFERENCE TO THE PHENOMENON OF SUPERSATURATION

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From the Laboratory of the London County Council.

(*Received January 1st, 1923.*)

The observations recorded below were made as part of a general investigation of the River Thames undertaken primarily from a public health point of view and the phenomena have only been investigated so far as was compatible with the main objects of the scheme. Casual examination of the water of the estuary of the Thames in the summer of 1914, in the course of which determinations of dissolved oxygen and chlorions were made, yielded several results in which the proportion of dissolved oxygen was much greater than was required for saturation with atmospheric oxygen of water of the observed salinity. Systematic observations made in subsequent years showed that supersaturation with dissolved oxygen occurred to a greater or less extent in each spring and in a less systematic manner later in the year.

Until recently we have found no direct references in scientific literature to this phenomenon of supersaturation in sea water, although it has been observed and its cause properly assigned in fresh water. One of us has pointed out [Coste, 1917] that "supersaturation—at any rate with oxygen—is by no means rare." It is sometimes to be attributed to "rapid variations of temperature," but "more often to its excretion from vegetation as a residual product of photosynthesis." In this paper it is noted that Morren (1843), who investigated the gaseous content of the surface water near St Malo, found that the oxygen content of sea water was greatest and the carbon dioxide least in bright weather. It should also be noted that the New York Harbour Board investigators recorded several cases where the volume of dissolved oxygen found was greater than that required for saturation, although they refrained from recording a greater percentage saturation than 100.

Recently Dr J. H. Orton, with whom we have been in correspondence, called our attention to a paper by Schulz [1922] in which he records and discusses cases of supersaturation with dissolved oxygen in the North Sea. Schulz refers to an earlier paper by Gehrke [1916] in which supersaturations up to 16 % are recorded.

Our own observations show very much greater excesses of oxygen than are mentioned by these workers, and as they are part of a long and systematic series are, we think, worth recording.

The observations, 1914–1922.

This investigation was not undertaken as part of an oceanographic scheme and the methods chosen were considered suitable for the purpose in view. Dissolved oxygen was determined by Winkler's manganous process which is recognised as most trustworthy. In some cases, dissolved oxygen and nitrogen were determined by Winkler's gasometric process of evolution by CO_2 generated in the water from calcite and hydrochloric acid. Latterly, hydrion concentration has been determined as described by Prideaux [1919], a simple nomogram being used to work out the ratio R.

Supersaturation was observed on various occasions in water collected in the navigable channel of the Thames at the following eight stations, which are named in order of sequence proceeding down river seawards: Mucking Light, Chapman Light, Southend, Nore Light Ship, West Oaze Buoy, Knob Buoy, Knock John Buoy, Edinburgh Lightship.

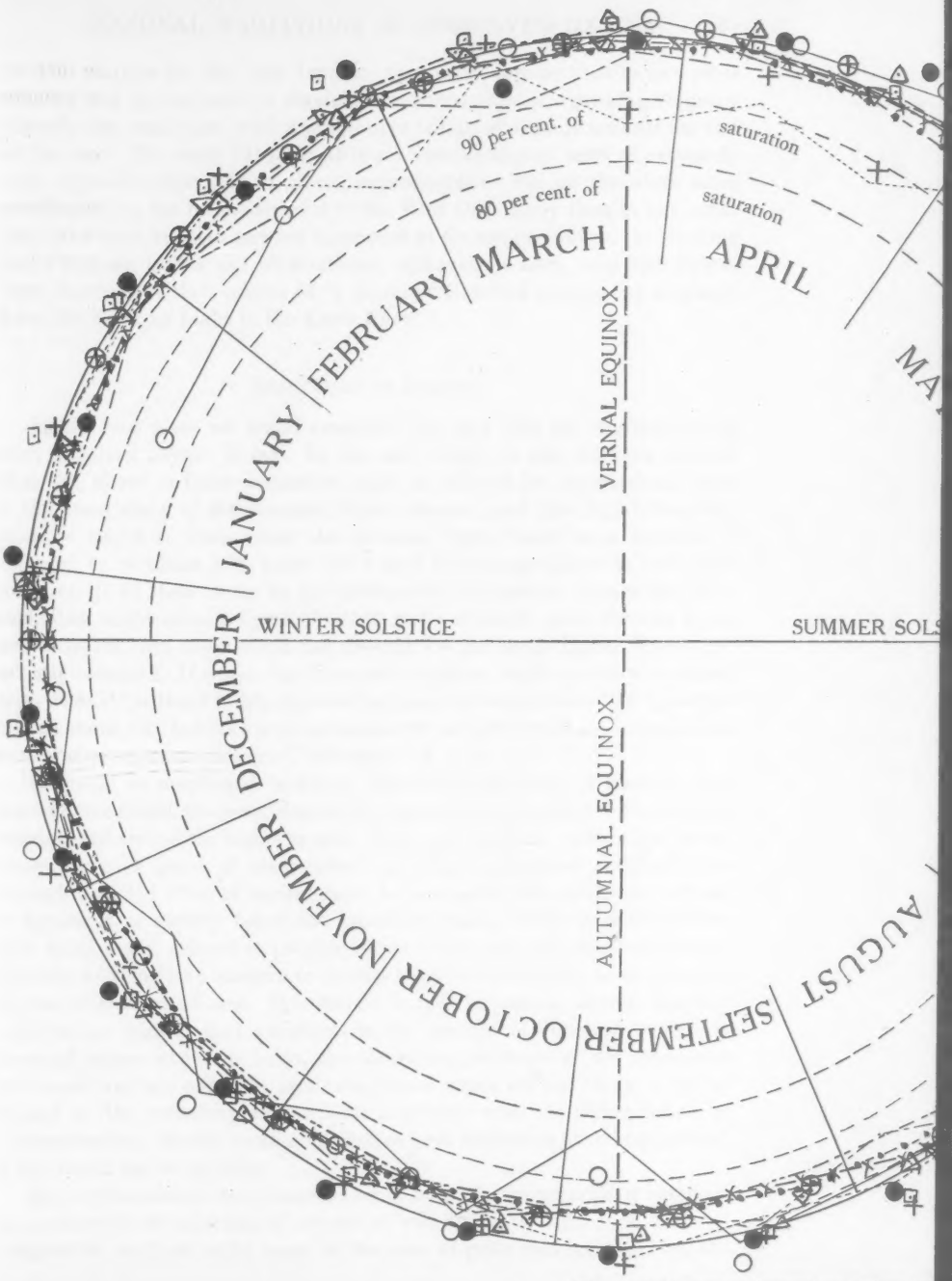
It is obviously impossible to present in tabular form the whole of the results obtained in connection with the one point of supersaturation with oxygen, but the maxima for each year for all the places are shown in Table I and the whole of the results obtained at one of the stations—the Knock John Buoy—are shown graphically in the polar diagram, Fig. 1. This form has been chosen as being suitable for showing the cyclic character of the variations, and at the same time enabling the results to be plotted as a continuum.

Small excesses of dissolved oxygen over the amount required for saturation have been observed in every month of the year (except October) but in most years excesses greater than 4 % of saturation have been confined to the months of May–August inclusive. Table I shows the time-incidence of supersaturation in each of the years under consideration. It is evident that supersaturation reaches a very pronounced maximum in May and June and that before May the excesses are very small.

A consideration of the maxima shows this.

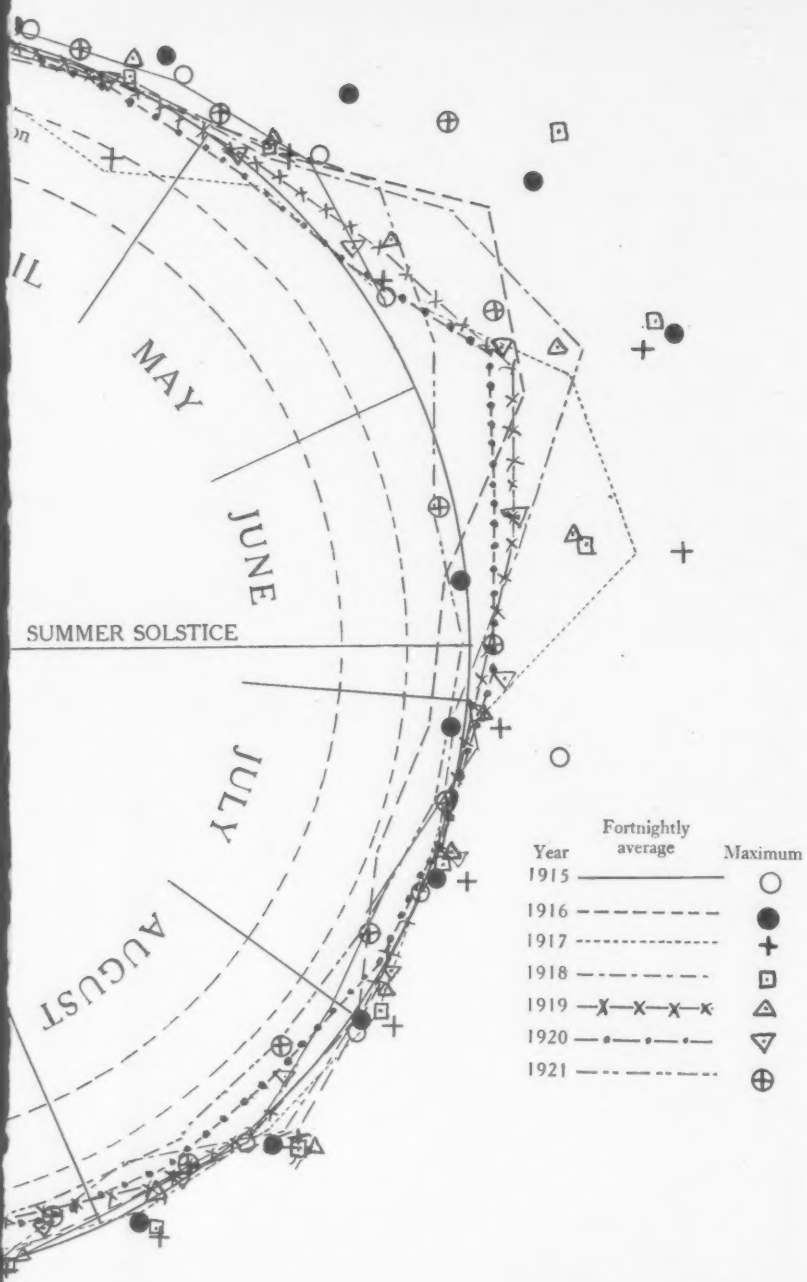
Table I. *Maximum percentage saturation in the months of each year.*

	1915	1916	1917	1918	1919	1920	1921	1922	General maximum
Jan.	—	104·9	—	104·1	—	—	—	—	104·9
Feb.	—	105·1	—	104·2	—	—	—	—	105·1
Mar.	—	—	—	107·1	104·5	—	—	—	107·1
Apr.	105·9	107·3	104·1	—	—	—	—	—	107·3
May	109·3	179·3	110·5	141·8	126·9	106·5	135·5	116·3	179·3
June	—	156·8	171·9	177·5	127·3	127·0	119·1	115·1	177·5
July	116·1	110·4	107·6	109·7	107·5	111·4	118·9	113·8	118·9
Aug.	104·8	110·2	108·2	111·4	113·5	—	—	—	113·5
Sept.	109·5	—	105·1	—	104·2	—	—	—	109·5
Oct.	—	—	—	—	—	—	—	—	—
Nov.	105·0	107·5	—	—	—	—	—	—	107·5
Dec.	105·9	—	—	—	—	—	—	—	105·9



NOTE. There were no observations in June 191

Fig. 1. Polar diagram showing the percentage saturation with dissolved oxygen of Estuary Water off the Knock



servations in June 1915.

Water off the Knock John Buoy for fortnightly periods in the years 1915–1921 inclusive.

The maxima for May and June are very much greater than in any other months, and the sequence of maxima shows that there is a growing tendency towards this maximum, with a progressive falling off from it towards the end of the year. The years 1916 and 1918 are outstanding as years of extremely high supersaturation. As to places, supersaturation was on the whole more pronounced in the neighbourhood of the West Oaze Buoy than in any other place and least frequent and less in amount at the upper stations, the Mucking and Chapman Lights and off Southend, although in 1916, very high figures were observed in May—above 50 % excess of dissolved oxygen—at all places from the Mucking Light to the Knob Buoy.

Discussion of results.

In the first place we would emphasise the fact that the supersaturation with dissolved oxygen is real. In the early stages of this work we realised that 2 % above or below saturation might be allowed for experimental error + the uncertainty of the standard figure adopted, and that high barometric pressure might at times cause the standard figure based on a pressure of 760 mm. to be about 2 % below the actual figure appropriate to such high pressure. If all these errors by an unfortunate combination were of the same sign, they might cause a figure of 104 % to be obtained when the true figure was only 100, but they would not account for the much higher figures frequently obtained. If water, say from polar regions, could somehow be raised from 0° to 15° without losing dissolved oxygen, this would raise 100 % saturation to about 140, but such supersaturation of oxygen would be accompanied by a similar supersaturation of "nitrogen" (N + A).

It should be mentioned, however, that while this range of possible error must be recognised, the lower degrees of supersaturation conformed to the rule which characterised the higher ranges. They were uniform—with minor variations—for each point of observation and they represented a definite rise through a period often of several days, as contrasted with prolonged returns of figures at or slightly below the saturation point. While admitting them with caution and subject to possible errors which have not been eliminated, it would we think be a mistake to dismiss them as due entirely to the possible sources of error mentioned. It is desired to note in passing, merely, that our observations suggest that variations in the amount of dissolved oxygen in estuarial waters above or below the saturation point are of not infrequent occurrence and are observed under conditions which are not likely to be explained by the periodic photosynthesis associated with the higher figures of supersaturation. Similar small divergencies from saturation have been noticed in the North Sea by Schultz.

Any explanation of the phenomenon of natural supersaturation based on the assumption of retention of oxygen in water recently subject to a rise of temperature such as might occur in the case of polar currents reaching the

North Sea fails, as will be seen from the following observations, to account for all the facts. Using Winkler's gasometric method, E. T. Butler and F. J. East in 1917 found that the proportion of nitrogen in water supersaturated with oxygen was normal, *i.e.* was approximately equivalent to that required for nitrogen saturation at the observed temperature. One result where the proportion of oxygen was rather less than 100 % is included in the table.

Table II. *Showing the percentage saturation of estuary water with oxygen and nitrogen in June*, 1917.

Place	Date	Temp.	Cl.	Dissolved gases cc. per litre		Percentage saturation	
				Oxygen	Nitrogen	Oxygen	Nitrogen
West Oaze	June 12	17·7°	1860	8·1	11·1	134	104
Nore	„ 14	18·0°	1845	7·9	11·0	150	104
„	„ 15	18·6°	1860	8·5	10·7	162	102
„	„ 18	19·7°	1870	7·44	10·7	145	104
Chapman	„ 19	20·3°	1505	4·89	11·0	90	104
West Oaze	„ 22	19·10°	1875	6·13	10·5	119	101

The one obvious explanation of supersaturation with oxygen only in spring is to be found in the normal vernal phenomena. An increased duration of daylight and greater penetrating power of the sun's rays lead to the rapid growth of the varied algal flora of the sea [cf. Moore, 1921] and thus to photosynthesis. The rising temperature of the water increases the velocity of photosynthesis as it does of other chemical reactions. This point has been investigated by Osterhout and Haas [1919] who found the temperature coefficient to be about 1·81 per 10° between 17° and 27°.

That water should become and remain appreciably supersaturated with a gas whilst it is in free contact with air is at first startling, and as we have indicated, we were at pains to satisfy ourselves of the reality of the phenomenon.

It is obvious that, given a source of pure oxygen (such as a growing plant) in water, the water around this source will take up the oxygen very rapidly if it is generated all over (or within) the surface of contact between water and plant without the formation of a limited interface which would occur if bubbles were generated. It would in fact appear that there will be no tendency to form bubbles until the concentration of dissolved oxygen is nearly five times as much as in water in equilibrium with air, since it is pure oxygen and not air which is evolved by the plant. Hence very little loss by reason of non-absorption by the water of oxygen generated is likely to occur. Loss may, however, occur in two ways: (1) by combination of the oxygen with oxidisable matter either with or without the aid of living organisms; (2) by escape into the air at the interface of air and water, following an upward diffusion of oxygen in solution. The amount of oxygen which can be liberated by photosynthesis is limited on the one hand by the relative abundance of chlorophyllous organisms and, on the other, by the available carbonic acid. Prideaux, Moore

and Herdman [1915] have shown that in sea water the removal of CO_2 from bicarbonate by a green algal growth can go on until the transformation

$$R(HCO_3)_2 \rightleftharpoons RCO_3 + H_2O + CO_2$$

is complete, but not beyond this point. This represents a p_H of about 9·1.

Sea water contains about 2·4 milliequivalents per litre of "uncompensated" base mostly as bicarbonate. If it be considered as all present as bicarbonate, then 2·4 milliequivalents of CO_2 are available for liberation and transformation into organic compounds and oxygen. The evolution of oxygen has been shown by Schulz [1922] to be equivalent within small limits to the CO_2 transformed. It is therefore possible in one litre of sea water to liberate 1·2 millimols. of oxygen or about 27 cc. of oxygen at N.T.P. This is from four to five times the amount required to saturate sea water in equilibrium with air. It is, in fact, almost enough to saturate it if under an atmosphere of pure oxygen. Further, the sea water is usually approximately saturated (with respect to the 21 % of oxygen in air) already, and also an increase of the alkalinity of sea water will cause it to take up more CO_2 (from which more oxygen can be liberated) from the air. There is, therefore, no difficulty in accounting for the liberation of oxygen in the required amount, given the presence of quick-growing organisms. It is reasonable, however, to expect that this liberation of oxygen should be accompanied by a decreased hydrion concentration.

Casual determinations of hydrion concentration during 1920 showed that there was a seasonal alteration of the kind expected (Table III). The results expressed as p_H, *i.e.* Briggsian log. of reciprocal of hydrion concentration—were as follows for sets of samples in March, April, May and June, 1920.

Table III. *Hydrion concentration of river water. Spring and early summer*, 1920. (*Expressed as* p_H.)

	Mar. 26	Apr. 22	May 5	June 7	June 9
Erith	7·78	7·78	about	7·76	7·69
Greenhithe	7·78	7·76	7·76	7·78	7·69
Gravesend	7·75	7·78	or less	7·78	7·69
Mucking Light	7·88	7·78	7·78	8·08	8·08
Chapman Light	—	7·78	7·88	8·21	8·20
Southend	7·88	7·88	8·20	8·41	8·31
Nore Light	7·94	7·88	8·20	8·41	8·31
West Oaze Buoy	7·94	7·94	8·20	8·41	8·30
Knock John Buoy	7·94	7·94	8·20	8·41	8·31
Edinburgh Light	7·94	7·94	8·20	8·31	8·20

It will be noted how the real alkalinity, as shown by hydrion concentration, on the whole increased at stations from the Chapman Light downward from March to June.

From June 1921, a regular series of observations was made, using Prideaux's method of double titration [1919]. The results of these are set out for one typical place in the estuary—the Knock John Buoy in Fig. 2, which shows the variation in p_H (hydrion concentration) for the period in question. It will be seen that during a great part of the period—from July 1921 to April 1922—

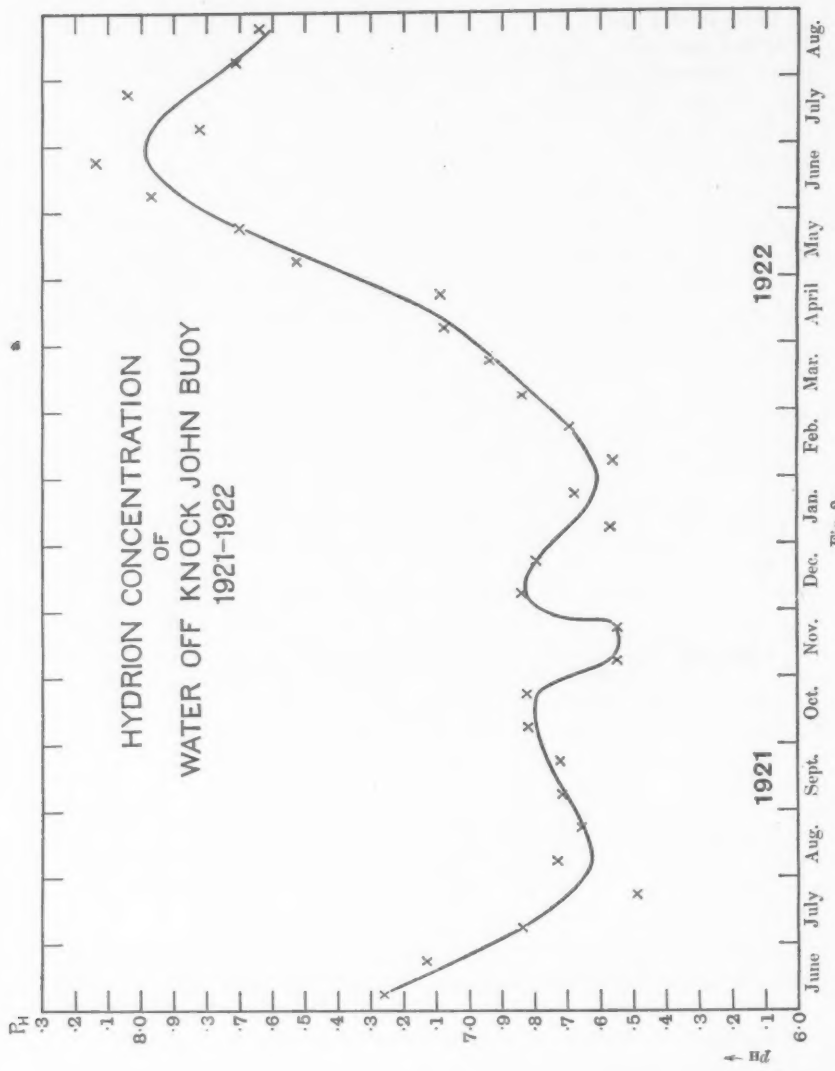

HYDRION CONCENTRATION
OF
WATER OFF KNOCK JOHN BUOY
1921–1922
P_H
8·3
·2
·1
8·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
7·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
6·0
P_H
1921
1922
June July Aug. Sept. Oct. Nov. Dec. Jan. Feb. Mar. April May June July Aug.

Fig. 2.

the water was acid, becoming from April 1922 to June 1922 more and more alkaline, with some indications of a falling off from June. Further the water was alkaline at the beginning of the period—June 1921.

These figures agree with the argument just developed that marked alkalinity and supersaturation should go on together, or rather that if supersaturation is observed, it should be at periods of high alkalinity (low hydrion), the latter being a more lasting record of photosynthesis than supersaturation.

There is some positive evidence of an increase of microscopical green organisms in the estuary water towards summer. It was obviously impossible to include in an investigation undertaken from a public health point of view, a full examination of microplankton but a regular microscopical examination of the sediment from the water was begun in July 1921 and the results returned, in respect of light green growths, dark (blue) green growths and diatoms, as trace (1), few (2), many (3), chiefly (4). The numbers attached were used to represent approximate orders of magnitude, and as nearly all the observations were made by one worker (Mr E. T. Butler) they should be sufficient to indicate a general increase or decrease of green organisms. Fig. 3 shows in graphic form the seasonal variation which, on the whole, follows the course suggested in this discussion.

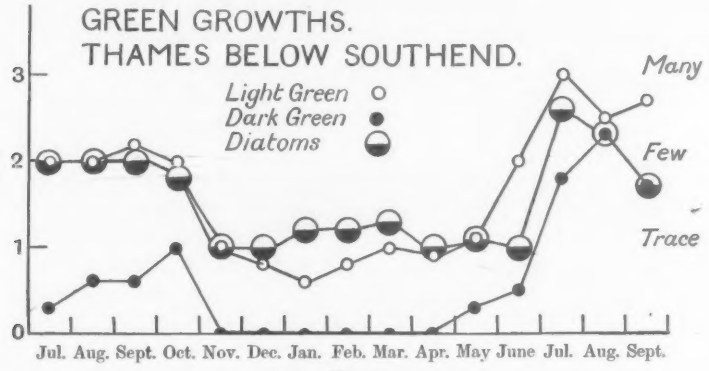


Fig. 3.

Sir W. A. Herdman [1922] has discussed "The distribution of the plankton as a whole and of its various constituents throughout the year" in the Irish sea. He says "The spring maximum of phyto-plankton starting in March, when the sea has still a low temperature and increasing to a climax in April, May or June; the diatom minimum at the height of summer in July or August; the secondary lesser maximum in autumn (generally September or October), very variable both in extent and in constituent organisms; and finally, the winter minimum have been recorded for every year and need no further demonstration."

The interaction between a gas (such as air) and a liquid in which it is soluble (such as water) is only a special case of heterogeneous reaction. It has been discussed generally by one of us [Coste, 1917] and the side of the equilibrium where the liquid is supersaturated with the gas was investigated by C. Bohr for solutions of carbonic acid [1899]. The case of unsaturation has recently been carefully investigated by Adeney and Becker [1919, 1, 2]. The general equation for the course of this action when the liquid is of uniform composition below the surface

$$\frac{C-x_t}{C-x_0}=e^{-kt} \text{ whence } \frac{dx}{dt}=k(C-x)$$

where C = the concentration corresponding to saturation, x_0 the initial concentration, x_t the concentration at any period of time t from the beginning of the action and k a constant which is really the evasion constant (a multiple of the *coefficient of evasion* of Bohr) is applicable to cases of supersaturation as well as to unsaturation, the only difference being that $C-x$ has a negative value both above and below the line on the left of the equation, as also has dx. Since this general equation implies that the rate of approach to saturation is, all other conditions being similar, proportional to the difference from saturation, it follows that around saturation, towards which the value converges, the rate of exchange becomes very slow, whether the liquid is supersaturated or unsaturated with the gas. The value of k will be small if the motion of water or air is slight, but it will increase rapidly if the molecules at the interface are rapidly changed, or if the area is enlarged. Hence still weather, when the motion of air and water is small, will tend to preserve supersaturation, whilst rough weather, when both air and water are in rapid motion, and waves, spray and air bubbles increase the interfacial area, will tend to bring about mere saturation, that is, stable equilibrium more rapidly. The effect of windy weather in increasing the dissolved oxygen in unsaturated water has been observed by R. H. Gould [1922] in the water of the Arthur Kill, New York Harbour. Our own observations on supersaturated water conform to those of these observers. Supersaturations in the estuary disappear rapidly under agitated conditions of the water, due to wind, and the comparatively low supersaturations observed in the years 1919–1922 are not improbably due to the coincidence of disturbed weather with the period of highest photosynthetic activity.

In still weather, the vertical movement of water due to convection by reason of the cooling and concentration of salts in the surface layer by evaporation is at a minimum and exchanges of dissolved gas (oxygen produced under the surface) between layers of water in different vertical planes will proceed more nearly by pure diffusion according to Fick's law [1855] causing a gradient of concentration, and less by the more efficient process of mixing. The considerable depth of the sea renders the formation of a gradient more easily possible.

Two experiments made by us illustrate the slowness of escape of oxygen from still water and the accelerating effect of agitation.

1. A large Winchester bottle was filled with tap water and inverted over water so that about $\frac{1}{2}$ litre of oxygen could replace some of the water. The bottle was stoppered and shaken repeatedly, and then left inverted with a water seal all night. Next day the water was siphoned off into another bottle which was filled to the neck. At intervals, samples were removed with suitable precautions for the determination of dissolved oxygen. The first three samples were taken within one hour and as the surface exposed was small, the loss of dissolved oxygen, although distinct, was small. The water level then reached the shoulder of the bottle and the surface exposed after this was practically constant. The results are shown in Table IV.

Table IV.

Time (hours)	0	$\frac{1}{2}$	1	3	47		51	52	144
Temperature	16·8	16·85	17·0	17·3	17·8	17·8[1]	18·9	19·4	16·0
Dissolved oxygen cc. per litre	24·4	24·4	24·3	24·2	11·44	10·8	11·12	10·8	6·36
% saturation	351	351	355	356	173	151	171	168	92·0

[1] Taken at top of water. All others siphoned from bottom. Value of k calculated from 47 hours = 0·026.

These results show how slowly still water parts with an excess of oxygen and those for 47 hours illustrate the working of Fick's law. The last result is to be explained by temperature changes. Barometric variations were considered in calculating the saturation values.

2. Similarly prepared water was placed in a cylindrical glass jar, exposing a surface of 95 cm.^{2} and stirred with a spiral glass stirrer at such a rate as not to break the surface. The results are stated in Table V.

Table V.

Time (hours)	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	2	3	4
Temperature	16·6	16·7	16·6	16·6	16·6	16·8	17·1	17·4
Dissolved oxygen cc. per litre	23·34	21·56	19·38	18·2	16·95	13·38	10·05	8·85
% saturation	338	312	281	264	253	195	148	127·5

Value of k about 0·5.

The effect of stirring in accelerating the exchanges between air and water is very marked, the value of the evasion constant being increased about 20 times [cf. Becker, 1921]. The experiment illustrates the effect of rough weather in rapidly dissipating supersaturation of sea water with oxygen or indeed with any dissolved gas.

It may be remarked that whereas the water of such rivers as the Thames or Seine is always much supersaturated with carbonic acid, that is, with calcium bicarbonate—in sea water which is much more agitated and mixed with the air the equilibrium is more closely approached [cf. Prideaux, 1919].

If what we have observed in the Thames estuary is true generally, not only of the seas around our coasts but also of the oceans beyond, the phenomenon is of more than academic interest. The vernal supersaturation of sea water, which from the nature of our observations was necessarily striking, is

only an index of a process which cannot fail to have results in much wider and unexplored fields. The seas at these times are a great natural factory where free oxygen is produced on a scale which, having regard to the relative areas of sea and land, may conceivably exceed that of land vegetation. The oxygen of supersaturation is but a proportion and probably a small one, of the volume set free by marine photosynthesis. It is tempting to speculate on the part played by these gases in the contribution to the salubrity of sea air. The tidal convection of oxygen during periods of supersaturation is no negligible datum among the purifying factors of polluted estuarial waters. The seas breaking on the shores at these times are liberating their surcharge of oxygen in its most active state and it is seen that not the chemist or the marine biologist alone is interested in a phenomenon which has for its theatre of operations the vast tracts of oceans and the littorals of continents.

It has always been realised that the sea acts as a great stabiliser of the CO_2 content of the air by reason of the bicarbonate-carbonate equilibrium. It appears that it is equally efficacious in the positive work of keeping up by continual contributions a steady proportion of oxygen in the air.

We have mentioned various co-workers in the body of the paper. We wish particularly to acknowledge the work of our colleagues Messrs E. R. Andrews, J. W. H. Biggs and E. T. Butler, the latter of whom carried out most of the work afloat. They have all, often under circumstances of difficulty, discomfort, and even danger, in a variety of ways, contributed to the elucidation of the points now set forth. In the conduct of the observations their work has been indispensable, and the accumulated data essential to an adequate survey of the phenomenon are a tribute to the assiduity of their co-operation.

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VIII. THE EFFECT OF CARBON DIOXIDE AND ACETIC ACID ON THE OSMOTIC PRESSURE OF HAEMOGLOBIN.

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The work of Martin [1896], Reid [1905], Hüfner and Gansser [1907], and Roaf [1910] has demonstrated that solutions of haemoglobin, as free as possible from electrolytes, have a measurable osmotic pressure. Measurements of osmotic pressure, therefore, offer a means of studying various changes in the physical and chemical state of haemoglobin in solution.

The purity of preparations studied in this way is, of course, of fundamental importance. Since haemoglobin is believed to exist in the blood as a sodium salt, there is the possibility that it may be isolated as the sodium salt rather than as the free acid. In order to remove any base which may be present, the solutions can be dialysed against water saturated with CO_2, as used by Bayliss [1911] in connection with his experiments on Congo-red. On carrying out such a procedure in an osmometer, preparatory to certain experiments, the osmotic pressure was observed to rise to a height several times greater than its original value when distilled water alone constituted the outer fluid. When the CO_2 solution was replaced by water the pressure fell to its former value. The present paper records a study of these changes, and similar changes caused by acetic acid.

There are, as far as I know, no observations recorded in the literature on the effect of CO_2 on the osmotic pressure of haemoglobin. Roaf [1910] reports experiments using laked corpuscles or a solution of once-crystallised, dried haemoglobin (to which alkali had been added to get it into solution) on the inside of the osmometer and acetic or hydrochloric acid as the outer fluid. In most cases when the acid used was strong enough to cause a rise in the osmotic pressure, the haemoglobin was changed to acid haematin, and the resulting increase in osmotic pressure must have been due, at least in part, to the increased number of osmotically active particles caused by the breaking up of the haemoglobin molecule. In two experiments (the only ones at all comparable to those reported below) methaemoglobin resulted from the treatment, and slight changes in osmotic pressure were observed. 0·001 *N* HCl gave an increase from 17·5 mm. (the control value against water) to 24·9 mm.

Hg and 0·005 *N* acetic acid gave an increase from 7·5 mm. to 11·2 mm. Hg. In neither case was the increase greater than 50 %, while in the experiments to be reported, a much greater rise in osmotic pressure was observed. No attempt was made to see whether or not the effect due to acids could be reversed by the substitution of water. Roaf concludes, on slight evidence [1910, p. 85] "It is most likely that the correct explanation is that haemoglobin forms ionising salts with alkalies and acids, and that the observed pressures are due to the sum of the pressure caused by haemoglobin and the ions into which the salt can split, for instance, sodium and "haemoglobyl" ions."

Preparation of haemoglobin.

The haemoglobin[1] was prepared according to the method described by Dudley and Evans [1921]. Well-washed corpuscles were laked by dialysing against distilled water in a collodion sac connected with a mercury manometer (see Hartridge [1917] for an earlier use of this method of laking corpuscles, to get a very concentrated solution of haemoglobin). Oxygen was bubbled through the thick haemoglobin solution after the stroma was removed by centrifuging. The crystals of oxyhaemoglobin which were formed in large amount by this procedure, were centrifuged from the mother-liquor and washed into a flask with one or two volumes of distilled water. By evacuating the flask, the oxyhaemoglobin was changed to the more soluble, reduced haemoglobin and the crystals passed into solution. Oxygen bubbled through this concentrated solution caused a recrystallisation of oxyhaemoglobin. Violent agitation of the flask when the oxygen is being bubbled through often helps to start crystallisation. This process may be repeated several times, though it is only fair to say that it becomes increasingly difficult to bring about this crystallisation. Perhaps a certain small percentage of inorganic salt is necessary for crystallisation, and this percentage may become reduced to a minimum as the purification continues. In these experiments, the haemoglobin preparations were always crystallised three, and usually four, times.

Great care was taken to prevent decomposition. The corpuscles were kept as cold as possible from the time of their shedding until the recrystallised haemoglobin was put into the osmometers (7–10 days). The NaCl used to wash the corpuscles was ice cold and the dialysis of the corpuscles, which took several days, was carried out in an ice box, at 4 to 7°. In the earlier experiments, the first crystallisation was brought about by the use of a minimal quantity of ice-cold alcohol added to corpuscles laked with an equal volume of water (the stroma removed, as in the other method, by centrifuging). The subsequent recrystallisation was carried out as given above. An experiment described on a subsequent page of this paper, shows that samples prepared

[1] The haemoglobin used in these experiments was prepared from the corpuscles of oxalated horse blood, obtained, in England, through the kindness of Dr R. A. O'Brien of the Wellcome Physiological Laboratories, and in the United States, through the kindness of Dr John A. Murphy of the Mulford Biological Laboratories.

in the two ways give the same osmotic pressure readings. On theoretical grounds, at any rate, the method of Dudley and Evans is certainly preferable, for alcohol, which may denature the protein, is not used.

Unfortunately, there is no very satisfactory way of determining the purity of a sample of haemoglobin. To test how far the recrystallisation removed the electrolytes from the haemoglobin solution, the conductivity of the mother-liquor (found to contain about 1 % haemoglobin) was tested after each crystallisation. For instance, in one preparation, the conductivity, determined roughly at room temperature (9·5°) was:

Mother-liquor above 1st crop of crystals						...	...	$1{\cdot}7 \times 10^{-3}$ mhos
,,	,,	2nd	,,	,,		...	...	$9{\cdot}5 \times 10^{-4}$,,
,,	,,	3rd	,,	,,		...	...	$2{\cdot}4 \times 10^{-4}$,,
,,	,,	4th	,,	,,		...	...	$6{\cdot}0 \times 10^{-5}$,,
A 13·8 % solution of haemoglobin from the 4th crystals								$2{\cdot}5 \times 10^{-5}$,,

Another preparation (1·96 % solution) had a conductivity of $3{\cdot}9 \times 10^{-5}$ mhos at 18°. This figure is practically identical with that given by Gamgee [1902] for purified haemoglobin. He gives $3{\cdot}25 \times 10^{-5}$ for a 2·24 % solution at 18°. The conductivity of the laboratory distilled water, not free from CO_2 at 18°, was $4{\cdot}25 \times 10^{-6}$.

If a saturated solution of haemoglobin is made up quickly, by adding distilled water to oxyhaemoglobin crystals, it is seldom stronger than 2 %. But if the packed haemoglobin crystals are allowed to stand, after removing the supernatant liquid, they will go into solution, sometimes not for several days, sometimes in the course of a few hours, even when kept on ice, giving a solution of 12 to 14 %. This solution gives a definite oxyhaemoglobin spectrum, but as it is difficult, spectroscopically, to distinguish reduced haemoglobin when a small amount of oxyhaemoglobin is present, it is not known how far reduction plays a part in this re-solution. Such a solution after several weeks' standing gives a definite spectrum of reduced haemoglobin. By dialysing corpuscles in collodion sacs against water, Hartridge [1917] was able to obtain solutions of haemoglobin as strong as 48 %. In speaking of the change in colour on dialysing, he says, p. 254: "This is due to the laking of the blood, and not to reduction of the haemoglobin, for if a thin film of the haemoglobin solution is removed from the dialysers it is a bright red in colour and analysis in Barcroft's apparatus shows that it is fully oxygenated." Further study is needed on this point. Both weak and strong solutions were used in these experiments.

Apparatus.

Osmometers of two types were used. Those which will be referred to as Type A were Moore and Roaf osmometers [1907], as modified by Bayliss [1909], with the exception that no precautions were taken to exclude the CO_2 of the air from the solutions in the osmometer. Type B osmometers were made of collodion sacs, fitted with U-tube mercury manometers of 1 mm. bore,

and suspended in bottles (about 150 cc. capacity) containing the outer fluid (Fig. 1). In order to use the minimum of solution (a large yield of haemoglobin does not result after so many crystallisations) and quickly to bring about an equilibrium between the inner contents of the osmometer and the outer fluid, use was made of a glass float which occupied almost the entire space within the collodion sac. Before sealing off the test-tube from which the float was made, mercury was admitted into the tube so that the float, when placed in the sac filled with haemoglobin solution, would slowly sink to the bottom of the sac. This prevented pressure on the bottom of the collodion sac and kept it from floating on the surface of the fluid and interfering with the opening into the manometer. Only 5 cc. of haemoglobin was then needed to fill the osmometer and there was only a thin film of solution between the glass float and the wall of the collodion sac, thus facilitating diffusion by doing away with the central core of liquid. Another advantage is that it is possible to see through such a thin film of solution, and note the appearance of any precipitate, change of colour, etc., in the solution in the osmometer. 10 % Anthony's negative cotton in equal parts of alcohol and ether was used to prepare the sacs.

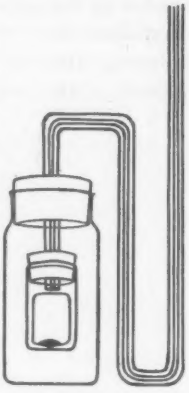


Fig. 1.
Type B Osmometer

Thymol and toluene were both found unsatisfactory for use in the osmometers or perfusing fluids, because after a time they caused precipitation of the haemoglobin. Haemoglobin solutions, however, used without any preservative did not undergo bacterial decomposition to any noticeable extent, even though kept for several weeks in the osmometers. Experiments were not continued any longer than necessary (usually less than two weeks) and in two cases osmometers were kept in an ice box at 4° throughout an experiment.

Briefly, the experiments were carried out as follows. The manometer tube was filled with haemoglobin above the mercury on the side to be connected with the collodion sac. The manometer carried a cork stopper which fitted into the reagent bottle containing the outer fluid of the osmometer. A float was placed in the collodion sac which was then filled with haemoglobin solution. A rubber stopper (with a hole for the manometer) was inserted securely, a rubber band placed around the neck of the sac and the joint covered with collodion. The manometer was then carefully inserted so that no air space was left in the system and the sac suspended in the bottle containing the outer fluid. The initial pressure in the manometer was due to the insertion of the manometer tube but this pressure rose or fell in a short time to the true osmotic pressure of the solution of haemoglobin. The osmometer and manometer could easily be removed from the bottle containing the outer fluid and held while changing the water in the bottle. Sufficient water was used completely to cover the collodion sac (about 100 cc.).

A comparison was made of preparations of haemoglobin obtained by the alcohol method and the Dudley and Evans method. Solutions of approximately the same strength (9 %) were placed in Type A osmometers and treated in the same manner in each case. The alcohol preparation had been crystallised four times and the D. and E. preparation three times. The following table will show that there is no appreciable difference in the osmotic pressure of the two solutions, either against distilled water or after treatment with acids.

Time of reading osmometers	Outer fluid	Osmotic pressure in mm. Hg: Alcohol preparation	Osmotic pressure in mm. Hg: D. and E. preparation
18. xi. 21 5 p.m.	Distilled water	39	52
19. xi. 21 1.40 p.m.	„ „	40	44
21. xi. 21 3.15 p.m.	Water + CO_2 (p_H = 4·0)	64	68
22. xi. 21 10.35 a.m.	„	76	81·5
23. xi. 21 12.40 p.m.	„	93	100
Later in same experiment:			
12. xii. 21 10.45 a.m.	Distilled water	52	54
13. xii. 21 11 a.m.	Acetic acid (p_H = 4·0)	68	72
14. xii. 21 4.20 p.m.	„ „	74	81
15. xii. 21 11.15 a.m.	Distilled water	70	76
16. xii. 21 10 a.m.	„ „	68	66

A comparison of oxyhaemoglobin and reduced haemoglobin was made. In this case the osmometers of Type B were used. The reduced haemoglobin was prepared from the same 2 % stock solution as the oxyhaemoglobin, by exposing a small amount of liquid to a vacuum for three and a half hours. In each case the pressure rose to practically the same extent (51–53 mm. Hg) with the use of CO_2. This was to have been expected, as the use of a saturated solution of CO_2 as an outer fluid probably changed the oxyhaemoglobin to the reduced form.

Experimental.

Below is given a detailed description of a characteristic experiment showing the influence of CO_2 and acetic acid on haemoglobin. A weak solution of haemoglobin was prepared by adding water to crystals of oxyhaemoglobin (four times crystallised) and centrifuging to get a clear solution free from crystals. By determining the percentage of solid and ash in 10 cc., it was found to be a 1 % solution of haemoglobin. Without delay, six osmometers of Type B were filled with the same solution. Distilled water of p_H 6·8 was used in all the osmometers as the outer fluid. On the second day of the experiment, and thereafter, oxygen was bubbled through the distilled water before using the osmometers, to keep the oxyhaemoglobin saturated with oxygen and to remove the CO_2 dissolved in the water from the air. When equilibrium had been obtained (the outer water having been changed frequently for two days), all six osmometers gave practically the same osmotic pressure (7 to 11 mm. Hg). This agrees quite well with the calculated osmotic pressure (10·9 mm.) of a 1 % haemoglobin solution at 20°, using 16,669 as the minimum calculated molecular weight of haemoglobin [Gamgee, 1898; Hüfner and Gansser, 1907;

Barcroft and Hill, 1910]. The experiments were divided into two groups. Three osmometers Nos. 22, 23 and 24 were used for group I, and the other three, Nos. 25, 26 and 27, for group II. One osmometer from each group (Nos. 22 and 25) was kept in an ice-box at 4° throughout almost the entire experiment. The data for these experiments are given graphically in Fig. 2 and Fig. 3.

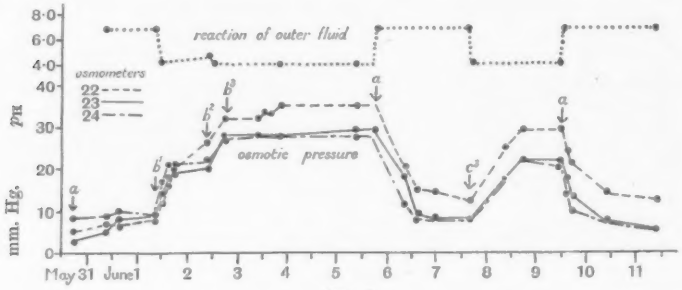


Fig. 2.

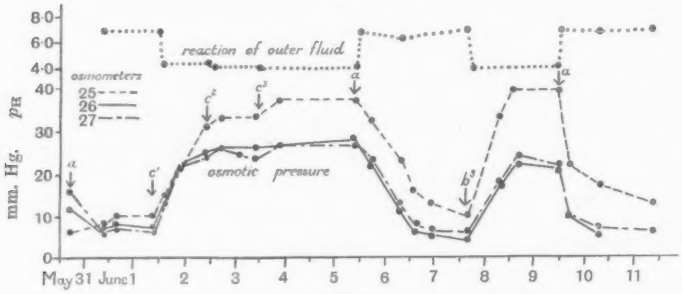


Fig. 3.

Figs. 2 and 3. Effect of CO_2 and acetic acid on the osmotic pressure of haemoglobin. Outer fluids:—a = distilled water (p_H = 6·8); b^1 = water 1/10 saturated with CO_2 (p_H = 4·2); b^2 = water half saturated with CO_2 (p_H = 4·0); b^3 = water fully saturated with CO_2 (p_H = 3·8); c^1 = acetic acid (p_H = 4·2); c^2 = acetic acid (p_H = 4·0); c^3 = acetic acid (p_H = 3·8). The arrows indicate the first introduction of a particular outer fluid which was changed several times a day until a new outer fluid was used.

In the osmometers of group I, water one-tenth saturated with CO_2 (p_H = 4·2) was used as outer fluid. This was made by adding nine parts of distilled water to one part of water saturated with CO_2. Care was taken to minimise the loss of CO_2 but no special precautions were used. The osmotic pressure rose to 20 and 22 mm. Hg in the osmometers at room temperature and to 26 mm. Hg in the one in the ice chest. Half saturated CO_2 solutions (p_H = 4·0) were used next, followed by fully saturated (p_H = 3·8), each of which caused a further increase of pressure as may be seen in Fig. 2. This pressure remained constant until, two days later, distilled water was substituted, causing the pressure to fall to its former value. Next, acetic acid of the

same p_H (3·8) as the last carbonic acid solution was used. Although the molar strength of the acetic acid was only 0·0013, compared with 0·042 *M* of the CO_2 solution, the osmotic pressure rose to practically the same extent in each case. This seems to indicate that this rise in osmotic pressure is due not to the particular acid used, but to the hydrogen-ion concentration of the solution. One experiment with 0·00037 *M* lactic acid ($p_H = 3·8$) gave similar results.

The order of the experiments was reversed with the second group of osmometers (see Fig. 3). After determining the osmotic pressure of the solution of haemoglobin against water, the p_H of the outer fluid was decreased as in the experiments in group I, using acetic acid instead of CO_2. The p_H of the solution was changed first to 4·2, then 4·0 and 3·8, with concentrations of acetic acid varying from about 0·0005 *N* to 0·0013 *N*. After maintaining this pressure for several days, distilled water was used to remove the acid and, finally, water saturated with CO_2 was put into the outer chamber of the osmometer. It can be seen from the figures that a rise in osmotic pressure occurs, and the rise is practically the same whether one acid or the other is used first as an outer fluid. In osmometer No. 25 the osmotic pressure, when CO_2 was used, was four times the value against water. The systems were considered to be in equilibrium when, after repeated changes of the outer fluid, the hydrogen-ion concentration became constant. The p_H of the outer fluid was determined colorimetrically, using bromophenol blue, bromocresol purple, and phenol red as indicators.

Results similar to those described above were obtained with a number of solutions of haemoglobin of different strengths using both types of osmometer. Haemoglobin prepared by both methods was used. With the stronger solutions the results are similar to those obtained with the 1 % solutions, but there was never proportionally as great a rise in the osmotic pressure produced by acids.

It is important to know whether or not the haemoglobin was decomposed or changed in any way during the experiments. There was no sign of decomposition other than the formation of a small amount of precipitated haemoglobin after acids were used. In the osmometers kept in the cold (Nos. 22 and 25) this amount was very slight. It was evidently too small appreciably to affect the osmotic pressure, for, whenever the solutions in these osmometers were dialysed against water, after the use of acids, the osmotic pressures returned to their former values. In the osmometers at room temperature, on the other hand, after repeated use of acids as the outer fluid, there was sufficient precipitation to account for a progressive reduction in the values against water, as, for example, in osmometer No. 24 from 9—8 to 5 mm. Hg.

To see whether or not oxyhaemoglobin changes on merely standing, two osmometers were filled with the same 1 % solution used in the experiments described in detail above, and were left standing, occasionally changing the distilled water which was used as the outer fluid. The solution at the end of several weeks was apparently unchanged. There was no precipitate and the solution gave the spectrum of oxyhaemoglobin.

Haemoglobin solutions, saturated with CO_2, are known to become reduced [Preyer, 1868]. But Heidelberger [1922] states that an oxyhaemoglobin solution will not become reduced when a mixture of 20 % oxygen and 80 % carbon dioxide is bubbled through the solution. In the experiments when water 1/10 saturated with CO_2 was used, there was probably enough oxygen present (18–19 %) to keep the haemoglobin completely oxygenated. When, however, water saturated with CO_2 was used, the oxyhaemoglobin was doubtless changed almost completely to reduced haemoglobin.

When air is again admitted to a solution of haemoglobin, through which CO_2 has been bubbled, methaemoglobin is formed [Preyer, 1868]. Oxyhaemoglobin treated with acetic acid [Menzies, 1895] is also changed to methaemoglobin. Hence it is not surprising that at the end of the experiments, after repeated use of CO_2 and acetic acid, the solution in the osmometers was found to give the spectrum of methaemoglobin.

Since a rise in osmotic pressure occurred in all the experiments when acids were used, whether or not oxyhaemoglobin, reduced haemoglobin, or methaemoglobin were present, evidently the effects of the acids are similar with each compound.

Discussion.

The increase in the osmotic pressure of a haemoglobin solution, when treated with CO_2 or with traces of acetic acid must, obviously, be due to the presence of a greater number of osmotically active particles than before. This might possibly be brought about in one of a number of different ways.

1. As the membrane is permeable to the molecules and ions of H_2CO_3 and acetic acid[1], they cannot exist in greater concentration inside the osmometer than outside it. Hence the acid used cannot be responsible for the rise in osmotic pressure.

2. If the haemoglobin had been aggregated in water and the acid had caused de-aggregation, there would have been an increase in osmotically active particles. Both assumptions are, however, contradicted by certain evidence. The observed osmotic pressure against water was approximately the same as the osmotic pressure calculated on the assumption that the haemoglobin existed as single molecules. During the course of the osmotic pressure experiments with acids, the gradual formation of a small amount of precipitate was observed in the osmometers, showing that a certain amount of aggregation had gone far enough to cause gross precipitation, and this would, of course, tend to cause a fall in osmotic pressure.

The work of Barcroft gives additional evidence on both these points. Barcroft [1914, Appendix II, pp. 314–315], gives the values for n in Hill's [1913] formula,

$$y = \frac{100kx^n}{1 + kx^n}$$

for the dissociation curves of dialysed haemoglobin at 40°, equilibrated at

[1] For the sake of brevity, since the results were the same with CO_2 and acetic acid of the same p_H, the discussion will hereafter be confined to the experiments with CO_2.

various CO_2 tensions. At 0 mm. CO_2 tension $n = 1$, at 8 mm. $n = 1{\cdot}78$, at 33 mm. $n = 2{\cdot}5$, and at 67 mm. $n = 2{\cdot}7$. Barcroft [1913, p. 481] gives "the average number of molecules of haemoglobin in each aggregate" as the definition of "n." According to these calculations, therefore, haemoglobin exists as single molecules in pure watery solution and is aggregated by CO_2[1].

Although, in the experiments described in detail in this paper, a weak solution of haemoglobin (1 %), dialysed against distilled water gave an osmotic pressure practically identical with the calculated value, stronger solutions (2–14 %) frequently gave lower pressures than those calculated for the particular concentration of solution used, indicating that some aggregation had taken place. Reid found values only one-third the calculated for 3–6 % haemoglobin solutions, and concludes [1905, p. 18] "that the molecular weight in solution in water would appear to be a multiple of the minimum calculated from analysis." Aggregation seems a more probable explanation.

In this connection it is interesting to note that Lillie found a fourfold increase in the osmotic pressure of a gelatin solution, when sufficient HCl was added to the inner and outer chambers of the osmometer, to make $M/770$ HCl solutions. He says [1908, p. 140] "The increased pressure may safely be regarded as due to a simple change in aggregation state." He does not suggest ionisation as a possible cause. Either de-aggregation or ionisation would give an increased osmotic pressure. The osmotic pressure of his gelatin solutions returned to their former values if the acid was removed by dialysis against water. There was no increase, and sometimes even a decrease, when egg albumin was treated similarly.

3. Decomposition of the haemoglobin into smaller molecules, which were still too large to pass through the collodion sac, would give an increased number of osmotically active particles. The fact that the osmotic pressure can be brought back practically to its former value (see especially No. 25), by substituting water for the acid, is evidence that decomposition plays little or no part in the rise of osmotic pressure. Haemoglobin decomposed into haematin and globin, for instance, would not re-unite to form haemoglobin by merely substituting water for acid in the outer chamber of the osmometer.

4. Martin [1896] has shown that the osmotic pressure of haemoglobin varies with the absolute temperature. Roaf [1912] has shown that the osmotic pressure of haemolysed pig's corpuscles rises from 6·9 mm. (maximum pressure per 1 % of organic matter at 16°) to 10·5 mm. Hg at 37°. Thus, even with wide variations in temperature the variation in osmotic pressure is not great. Furthermore, in the experiments reported in this paper, the temperature seldom varied more than a few degrees, and in no case was there parallelism between a rise of temperature and a rise in osmotic pressure.

[1] *Note added Feb. 5th*, 1923. This aggregation should cause a lowering of osmotic pressure. The rise of osmotic pressure, which is observed in these experiments, must occur in spite of the aggregation caused by the CO_2. The rise in osmotic pressure cannot, therefore, be as great as it would have been had there been no aggregation.

5. There still remains the possibility of the formation of ions by (*a*) ionisation of haemoglobin bicarbonate or (*b*) ionisation of the haemoglobin molecule into protein ions. These possibilities the author now proposes to discuss in some detail.

(*a*) Since haemoglobin is an amphoteric electrolyte it may be assumed that it can combine with acids to form salts. It has been shown that the osmotic pressure of haemoglobin against CO_2 may be nearly four times as high as against water. If haemoglobin bicarbonate is formed, the salt must have, under these circumstances, an average of three bicarbonate radicles to every haemoglobin radicle in order to furnish enough ions to bring about the increased pressure. A study of Donnan's theory of membrane equilibrium [1911] will show that there would be, under these circumstances, a counter osmotic pressure that must be taken into consideration. Donnan and Harris [1911] and Bayliss [1911] have shown that there is unequal distribution of an electrolyte, such as NaCl, between a solution of the sodium salt of Congo-red inside, and water outside, a membrane impermeable to the Congo-red. From thermodynamic considerations, Donnan [1911] showed that the product of the concentrations of the Na and Cl ions inside the membrane must equal the product of their concentrations outside the membrane. Since a part of the Na ions inside the membrane comes from the sodium salt of Congo-red, then the concentration of the ionised NaCl inside the membrane, will be less than that outside the membrane. This excess of NaCl outside gives rise to a counter osmotic pressure and therefore the osmotic pressure of the Congo-red salt will be less than the theoretical. The extent of the depression of the osmotic pressure depends on the relative concentrations of the colloid and electrolyte.

Applying this theory to our experiments, we have the following: where R = the cation of haemoglobin bicarbonate, (1) = outer fluid, (2) = inner fluid, and x, y and z are ion concentrations.

(2)	(1)
$\underset{z}{(R^{\cdot})}\ \underset{z}{(HCO_3')}$	
$\underset{y}{(H^{\cdot})}\ \underset{y}{(HCO_3')}$	$\underset{x}{(H^{\cdot})}\ \underset{x}{(HCO_3')}$
$(H_2CO_3) = (H_2CO_3)$	

According to Donnan and Allmand [1914] the concentration of the undissociated electrolyte is the same on both sides of the membrane, and hence will not affect the osmotic pressure in any way. The ionic equilibria may be expressed:

$$y\,(y + z) = x^2.$$

Under these circumstances, instead of the osmotic pressure being dependent only upon the concentration $2z$, there is a counter osmotic pressure represented by $2x - 2y$. Furthermore, the H-ion concentration (y), inside the membrane, is less than the hydrogen ion concentration (x), outside the membrane. The following calculations, made on the basis of the foregoing, show

the extent of the counter osmotic pressure and the difference in p_H on the two sides of the membrane. The haemoglobin bicarbonate is this time written with three bicarbonate radicles for each haemoglobin radicle to account for the four-fold increase of osmotic pressure observed while using CO_2.

$$\begin{array}{l|l} (R^{\cdot})\,(HCO_3')\,z & \\ z & \\ \quad (HCO_3')\,z & \\ \quad (HCO_3')\,z & \\ (H^{\cdot})\,(HCO_3') & \qquad (H^{\cdot})\,(HCO_3') \\ y \quad y \quad . & \qquad x \quad x \end{array}$$

$$y\,(y+3z)=x^2.$$

When the p_H of the outer fluid is 4, $x = 0{\cdot}0001\ N$. A 1 % haemoglobin solution (molecular weight 16,000) is $0{\cdot}0006\ M$. Assuming complete ionisation of the haemoglobin bicarbonate, $z = 0{\cdot}0006\ N$. Substituting in

$$y\,(y + 3z) = x^2,$$

$$y\,(y + 0{\cdot}0018\ N) = 0{\cdot}000{,}000{,}01\ N,$$

$$y^2 + 0{\cdot}0018\ N\ y = 0{\cdot}000{,}000{,}01\ N,$$

y^2 being infinitesimal as compared with the other values, may be disregarded. Hence

$$y = 0{\cdot}000{,}006\ N.$$

The counter osmotic pressure is dependent upon the excess of ionised H_2CO_3 outside the membrane over that inside the membrane

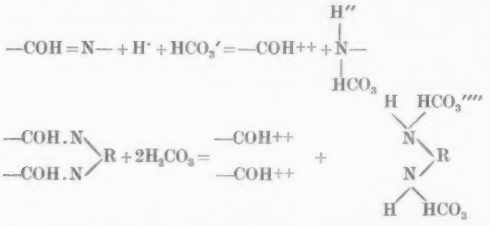
$$2x - 2y = 0{\cdot}000{,}18\ N.$$

The osmotic pressure of the haemoglobin bicarbonate, which is dependent upon the concentration $4z$ ($0{\cdot}0024\ N$), would, in this case, be reduced about 10 % due to the unequal distribution of the ionised H_2CO_3. For example, if the osmotic pressure had been 10 mm. Hg against water, and a haemoglobin bicarbonate were formed (such as described above), the pressure instead of rising to 40 mm. Hg would rise to 36 mm. Hg. In these experiments, therefore, since the x concentrations are much less than the z concentrations, the counter osmotic pressure does not interfere much with the osmotic pressure determinations. With stronger solutions of haemoglobin the counter osmotic pressure would be proportionately less. A 10 % solution would show 99 % of the calculated osmotic pressure.

The effect of the Donnan equilibrium on the H-ion concentration of the inner fluid is more marked in the example given above than is the effect on the osmotic pressure. y, the H-ion concentration of the inner fluid, is $0{\cdot}000{,}006\ N$ ($p_H = 5{\cdot}2$), while x, the H-ion concentration of the outer fluid is $0{\cdot}0001\ N$ ($p_H = 4{\cdot}0$). With a 10 % solution of haemoglobin, in the form of haemoglobin bicarbonate, the inner fluid would have a p_H of 6·2. Since the isoelectric point of haemoglobin is about p_H 7·0 (Michaelis and Takahashi [1910] give $1{\cdot}9 \times 10^{-7}$ ($p_H = 6{\cdot}75$) and Campbell and Poulton [1920] $p_H = 6{\cdot}98$), undoubtedly a salt could be formed at a p_H of 5·2. A p_H of 6·2 might also be sufficiently acid for salt formation, although Bayliss [1921] has shown, for

dialysed blood serum, that there is a considerable lag in salt formation with acids, until the p_H is well on the acid side of the isoelectric point.

(*b*) Considerable evidence for the dissociation of proteins into two or more protein ions, due to the action of acids and alkalies, is given by Robertson. He says [1918, p. 20] "we may now regard it as an established fact that some elements in the protein molecule other than the terminal $—NH_2$ or —COOH groups are responsible for the acid- and base-neutralising power which is possessed in such a marked degree by many proteins." Substituting H_2CO_3 for HCl in equations ii (p. 25) and vi (p. 26) we have:



In this case we would presumably have no Donnan effect, as the freely-diffusing dissociated carbonic acid has no ion in common with the haemoglobin, which has dissociated into protein ions. With the equal distribution of H and HCO_3 ions on both sides of the membrane there should be the same H-ion concentration on both sides. If the above theoretical considerations are sound, it should be possible to decide what kind of dissociation of haemoglobin has taken place by testing the p_H of the haemoglobin solutions in the osmometers, by means of the hydrogen electrode. If only protein ions are formed, the H-ion concentration of the inner fluid should be the same as the outer fluid. If haemoglobin bicarbonate is formed, and the Donnan equilibrium comes into play, the inner solution should be much less acid than the outer. The same relations should hold true for a haemoglobin acetate also. The author proposes to investigate these points shortly.

Conclusion.

It is shown that the osmotic pressure of solutions of purified haemoglobin is three or four times as great when dialysed against acetic acid, or water saturated with CO_2 (both about p_H 4), as against water alone. Reasons are given for believing that this is due to the formation of some sort of an ionising salt of haemoglobin, either one which ionises into several protein ions, or into protein and acetate or protein and bicarbonate ions.

This investigation was undertaken at the suggestion of Professor Sir William Bayliss, to whom the author wishes to express her thanks for encouragement and advice throughout the course of the work.

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IX. AMMONIA CONTENT OF THE BLOOD IN NEPHRITIS.

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In October, 1921, Nash and Benedict [1921] published a paper in which they gave an historical review of previous work concerning the ammonia-content of blood, together with work which they themselves had done on this subject, using dogs under various conditions. They showed that low figures, ranging from 0·03 to 0·14 mg. per 100 cc., were obtained for the blood-ammonia in normal cases, and that in nephrectomised dogs the values were markedly reduced. They also found a higher percentage of ammonia in blood from the renal vein than elsewhere in the circulation. On these data they put forward the view that the kidneys are the site of ammonia-formation in the body, the small quantities present in the blood being regarded as an overflow from these organs into the systemic circulation.

In view of this hypothesis it was considered that a series of estimations of the blood-ammonia in cases of severe nephritis would be of interest. In selecting the cases, preference was shown for those with a marked nitrogen-retention, as it was thought that these would be the most likely to give abnormal figures. In the series quoted other details relating to the renal efficiency of the patients are given wherever available.

Method.

The Folin-Denis aeration method was employed, as described by Nash and Benedict. With the exception of Case 3, the time elapsing between the drawing of the blood and the beginning of aeration was not greater than 10 minutes. In the case mentioned the interval was five minutes longer, and the sample was placed on ice during this time. An electric suction pump was used for the aeration, and in all cases this step in the analysis lasted 15 minutes.

Controls.

Controls for the method, as employed, were carried out as follows:

(1) 5 cc. of a standard ammonium chloride solution, containing 0·014 mg. N (or 0·28 mg. N %) were used for an analysis in place of 5 cc. of blood. The resulting solution in the acid-holding tube, when Nesslerised, gave an exact match with 5 cc. of the untreated standard simultaneously Nesslerised.

(2) A sample of blood from a patient without known renal disease (Case 1, Table II) was analysed, and the ammonia estimated immediately. Without further delay 0·5 cc. of diluted ammonium chloride solution, containing

0·014 mg. N was added to the aerated blood, and a second aeration immediately performed, adding another 1·0 cc. of carbonate-oxalate solution. The resulting solution in the acid-holding tube gave an exact match with the same quantity of diluted standard in 5 cc. of ammonia-free water, simultaneously Nesslerised. This experiment was repeated on Case 11, Table II, adding 0·5 cc. containing 0·0047 mg. N, with the same result.

(3) Since it was suggested that this result might have been obtained as the result of incomplete removal of the blood-ammonia during the first aeration, the following investigation was made:

5 cc. of freshly drawn blood (Case 2, Table II) were aerated, and the blood-ammonia immediately estimated. With the least possible delay, a second aeration was performed on the same blood for another 15 minutes. The fluid in the acid-holding tube showed no trace of colour on Nesslerisation.

This experiment demonstrated not only the adequacy of the method as a means of analysis, but the purity of our reagents with respect to ammonia.

It should be added that, where the concentration of blood-ammonia was under 0·04 mg. N %, the values given are only approximate, since the depth of colour on Nesslerisation was too faint to admit of accurate estimation.

Table I gives figures for a series of 17 cases examined in the wards of the hospital. Of these Cases 1, 5, 12 and 17 died, but in only the last two mentioned was a post-mortem obtained. The diagnosis of chronic interstitial nephritis was confirmed, but the picture was complicated in Case 12 by the additional finding of infective endocarditis, which probably superimposed an acute infection on the already damaged kidneys.

These same four cases presented symptoms clinically recognised as uraemia, *i.e.* pronounced drowsiness with irregular muscular twitchings, uraemic smell and irregularities in respiration. In Case 12 this irregularity was Cheyne-Stokes in type. Case 1 showed a urea frost during 48 hours before death, a rare phenomenon in which urea crystallises on the surface of the skin giving it a rough, whitish appearance. Case 13 is of considerable interest, since the patient was not confined to bed, but actively engaged in domestic service and not apparently inconvenienced by her renal inefficiency. In other respects the table is self-explanatory. In estimating p_H of blood and plasma bicarbonate the method of Van Slyke and Cullen was employed [1922]. Table II gives a series of 11 cases in which the blood-ammonia was estimated in cases with no evidence of renal disease. Cases 5, 6, 7 and 8 were normal people, the rest were patients suffering from the diseases indicated. The number of cases investigated in this second series is not sufficient to enable one to draw conclusions, but one is led to think that the ammonia-content of the blood in normal people is almost negligible, and that the blood of ill people contains amounts of ammonia which, though small, will admit of estimation. In this class the value obtained for the blood-ammonia seems to be independent of

Table I. *Values for blood-ammonia in cases with renal disease.*

Case	Diagnosis	Blood: Urea in mg. per cent.	Blood: NH_3 in mg. N per cent.	Blood: HCO_3	Blood: p_H	Urine: Volume 24 hours	Urine: p_H	Urine: $R - \frac{A}{N}$	Urine: Titratable acid in cc. of 0·1 *N* per cent.	Urine: Ammonia in cc. of 0·1 *N* per cent.	Urine: P.S.P. excreted in 1st 2 hours	Remarks
1	Chronic nephritis	480	0·044	44	7·35	1620	—	1·96	19·6	10	—	
		600	0·044	42	7·35	1920	4·8	2·1	18·8	9	0	4 days later
		600	0·088	46	7·4	1620	—	2·8	20	7	—	9 days later. Died
2	Chronic interstitial nephritis	66	0·068	68	7·55	—	6·8	—	—	—	—	
3	,, ,, ,,	41	0·044	—	—	—	—	—	—	35	—	15 mins. delay before aeration
4	Toxaemia of pregnancy	33	0·044	—	—	—	—	—	—	—	28	
5	Chronic interstitial nephritis	564	0·05	35	7·4	Alkaline. Bacterial action					0	Died
6	Chronic parenchymatous nephritis	120	0·044	61	7·4	1130	4·9	3·4	54·8	16	15·5	
7	Pyonephrosis	41	0·066	59	7·4	—	—	1·0	8	8	21·7	
8	Subacute	55	0·04	—	—	—	4·9	1·5	25	16	32	
9	Chronic interstitial nephritis	92	0·045	—	—	—	4·8	1·3	25	19	—	
10	Acute	120	0·066	67	—	Alkaline		—	—	—	—	
11	Chronic interstitial nephritis	63	0·044	—	—	—	—	3·1	34	11	11·5	
12	,, ,, ,,	171	0·088	43	7·6	610	Alkaline		—	—	Trace	
		240	0·044	54	7·4	—	—	—	—	—	—	5 days later. Died
13	Congenital bilateral cystic kidneys	141	Nil	33	7·2	—	—	0·16	2	12	Trace	Urine doubtful. Acid $)NH_3$ on previous occasions
14	Subacute	67	0·088	—	—	1300	—	4·9	39	8	16·8	
15	Chronic nephritis	171	0·09	62	7·4	1940	—	2·6	16	6	7·4	
16	Infective endocarditis with renal damage	63	0·02	46	7·6	570	5·6	0·34	14	41	39	
		52	0·03	42	7·5	—	—	—	—	—	—	7 days later
17	Chronic interstitial nephritis	343	Nil	13	7·2	—	Alkaline ? bacterial action				—	
		400	0·04	74	7·55	—	—	—	—	—	0	4 days later. Bicarbonate given in interval
		600	0·02	41	7·35	—	—	—	—	—	—	10 days later. Died

the nature of the illness, and the range of figures for the non-renal cases coincides with the range obtained for renal cases. More work along this line would be useful. It is possible that such factors as toxaemia might be found to play a part.

Table II. *Values for blood-ammonia in cases without renal disease.*

Case	Diagnosis	Blood-NH_3 in mg. N %	Remarks
1	Addison's disease	0·05	
2	Rheumatic fever	0·088	
3	Catarrhal jaundice	0·02	
4	Diabetes mellitus	0·02	No ketosis present
5	Normal	Very faint trace	Blood drawn in laboratory and transferred to pump immediately
6	,,	Nil	
7	,,	Faint trace	
	[repeated]	,, ,,	Stood 10 mins. before aeration
8	Normal	,, ,,	
9	Carcinoma of stomach	,, ,,	
10	? Duodenal ulcer	,, ,,	
11	Hepatic enlargement	0·05	

Discussion.

If we adopt the view that the ammonia present in the urine has been preformed in the blood and simply excreted by the kidneys, it seems legitimate to expect that cases with marked urea-retention would also show ammonia-retention to a comparable degree, particularly if the formation of urea from ammonia is a reversible reaction. If, however, Nash and Benedict [1921] are correct in their hypothesis that the kidneys are the site of ammonia-formation, we might reasonably suppose that there would be no ammonia-retention corresponding to high values of the blood urea, since ammonia-formation would be suppressed in common with other renal functions.

A consideration of Table I at once shows that no ammonia-retention has been found in any of the cases. The values obtained vary from 0·09 mg. N % to zero and are of the same order as those of the cases in Table II.

Next, if the blood-ammonia is not renal in origin, but is derived from other organs it might be expected, on the filtration hypothesis, that the ratio between urea and ammonia in the urine would bear a close relation to the same ratio in the blood.

Taking as normal figures:

Urea in urine	2000 mg. %.
Ammonia	33 mg. %.
Urea in blood	25 mg. %.

Then ammonia in blood $= \frac{33 \times 25}{2000} = 0{\cdot}4$ mg. %.

This figure is over four times as great as the maximum value observed.

Also, if ammonia is preformed in the blood, we ought to find a definite relation between the values for blood-ammonia and urinary ammonia. In case 16, the excretion of ammonia in a 24 hourly specimen was equivalent

to 57·4 mg. N %, whereas the blood-ammonia was only 0·02 mg. N %. Supposing her weight to be 50 kilos, and the volume of her blood 4,250 cc. (85 cc. per kilo) the total ammonia-content of her blood would be 0·85 mg. N. The whole volume of her blood must therefore circulate through the kidneys completely 384 times per 24 hours, and the kidneys must be 100 % efficient in removing all ammonia from the blood, in order to make up the figure for the urine ammonia. Or, in other words, the total blood flow through the kidneys must be 1632 litres per 24 hours. This is a possible rate of flow, according to Starling [1915], but it is highly improbable that a kidney with such impaired functions as are shown in this case could rise to a 100 % efficiency with respect to ammonia excretion.

Further evidence may be adduced from the consideration of the ammonia-content of urine and blood in acidosis. A large percentage of the total acidity of the urine is represented by ammonia as ammonium salts. This amounts, in normal persons, to about 30 mg. %. The ammonia in the blood, at a generous estimate, is present to the extent of 0·1 mg. %. But if ammonia is used as a neutralising agent for non-volatile acids in the blood it might be expected that, where increased acid is circulating, there would be a proportionate increase in the blood ammonia. Thus case 17 shows a marked acidosis with a decrease in the plasma bicarbonate (taking the normal as 60 vols. %) equal to 0·02 gram-equivalents per litre. If only a quarter of this were neutralised by ammonia (25 % being a low value for the percentage of acid neutralised by ammonia in urine) the figure for the blood-ammonia nitrogen would equal 0·005 gram-equivalents per litre = 0·005 × 14 g. N per litre = 0·007 g. N %. This is 70 times in excess of the maximum value obtained in any case in the series. Similarly, arguing on the same lines, the figure for Case 13 would be 0·004 g. ammonia N %, and in Case 5 it would be 0·0049 g. ammonia N %. Both of these are approximately 40 times in excess of the values found. It is perhaps noteworthy, in this connection, that Cases 17 and 13 are the only cases in Table I in which no trace of blood-ammonia could be found.

Summary.

1. Twenty-three estimations of the blood-ammonia for a series of 17 cases are recorded, in patients showing advanced renal disease. In all cases the values reported are under 0·1 mg. % and are comparable to the values recorded for eleven cases with no known renal disease.

2. Controls for the technique employed are described.

3. It is argued that the evidence available lends support to Nash and Benedict's hypothesis that the kidneys are the site of ammonia formation in the body, and that the traces found in the systemic circulation represent an overflow from these organs.

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X. TETHELIN:—A GROWTH-CONTROLLING SUBSTANCE OBTAINABLE FROM THE ANTERIOR LOBE OF THE PITUITARY BODY.

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(*Received January 5th, 1923.*)

In a recent communication, Drummond and Cannan [1922] have published an account of certain experiments and critical considerations which, in their estimation, invalidate the conclusions drawn by the author concerning the effects of pituitary (anterior lobe) substance upon the growth of animals [1916, 2, 3; Robertson and Ray, 1919] and the presence of a growth-controlling agent in the precipitate produced by ether from an alcoholic extract of the anterior lobe tissue (Tethelin).

The paper of Drummond and Cannan consists of two parts, the one dealing with the question of the composition of tethelin, and the other describing their failure to influence the growth of mice by the administration of pituitary (anterior lobe) tissue. These sections of their paper are presented as if they were logically related and mutually confirmatory, but in fact, save by the common bond of indiscriminate criticism, they are in no way connected. I propose to deal with the two parts separately in the order in which they are presented by Drummond and Cannan.

Isolation of tethelin.

In my original article describing the isolation of tethelin from the alcoholic extract of pituitary (anterior lobe) tissue [1916, 4] I was at pains to point out that the substance is exceedingly susceptible to oxidation. Drummond and Cannan declare that they "have made several attempts to prepare this substance and to obtain a white precipitate such as Robertson described by following the details of his method." This phraseology appears to be meant to create the impression that they failed. The obvious course for Drummond and Cannan to pursue would have been to procure the substance from the manufacturer[1], in which case they would have ascertained that others, besides myself, have been able to prepare this product without pronounced dis-

[1] H. K. Mulford and Co., Philadelphia.

coloration[1]. Instead of this, they introduced an "improvement" into my method and employed an atmosphere of carbon dioxide throughout its preparation. Now, as a matter of course, this was the first method which occurred to me to protect tethelin from oxidation, and it was abandoned because I found that carbon dioxide itself caused softening and darkening of the product. A possible reason for this became apparent later, when Schmidt and May [1917] found that heating of tethelin, even in faintly acid solutions, induces decomposition which may be rendered manifest by physiological tests[2].

From the material which they prepared in this manner Drummond and Cannan now endeavoured to separate several fractions in order to demonstrate that it was a mixture of substances. After hydrolysis by alkalies and by acids I also have separated various fractions from the hydrolysate, and these experiments are described in detail in my paper. After procuring three fractions which presented differing characteristics they abandoned further attempts, owing to their "conviction that the matter was not worthy of further consideration." In agreement with this conviction we may terminate our discussion of this portion of Drummond and Cannan's contribution.

Feeding experiments.

The question of the chemical unity or composition of tethelin is, of course, not in the least involved in the separate question whether or not it contains the whole or a portion of those substances in the pituitary gland which are capable of affecting the growth of tissues. This latter question has been approached in my experiments by the direct administration of tethelin to normal growing animals and to animals inoculated with carcinoma. Drummond and Cannan approach it by the administration of pituitary (anterior lobe) tissue to young mice. In their expressed belief their results invalidate conclusions which have been based by numerous observers besides myself upon experiments extending over many years upon a variety of animal types. They state in the first place that administration of the tissue of the anterior lobe of the pituitary body to animals is devoid of effect upon their growth, in contradiction to the previous findings of Schafer [1912], Cushing [1909], Aldrich [1912], Wulzen [1914], Maxwell [1916], Goetsch [1916], Marinus [1919], Pearl [1916], Uhlenhuth [1921, 1922], and Robertson and Ray [1916, 2, 1919, 1920], all of whom have reported more or less decided effects upon growth as the result of such administration. One would expect to find an opinion, advanced in opposition to such united testimony, supported by a wealth of experimental evidence and an exhaustive analysis of technical detail, but, on

[1] Under laboratory conditions discoloration may be totally avoided, but to attain this the alcohol and ether used must be neutral and anhydrous, the salts employed must be strictly neutral (we manufactured our own calcium sulphate for this purpose) and a heated and evacuated desiccator must be employed [Robertson and Schmidt, 1916].

[2] Doubtless an atmosphere of dry nitrogen would overcome these difficulties, but we have not found it necessary to resort to this.

the contrary, the evidence presented, in so far as any quantitative evidence is advanced at all, is derived from data furnished by the growth of *ten* animals of each sex during a period of administration which extended over nine weeks[1].

It is urged by Drummond and Cannan that the testimony of former observers is conflicting, in that some report acceleration of total growth as a result of administering pituitary tissue, while others report retardation. The testimony is not conflicting, however, if we view it in the light of the experiments of Schafer, Wulzen, Maxwell, and Robertson and Ray, which have shown that the retardation occurs at one age (and possibly affects one kind of tissue), while the acceleration occurs at another. In seeking to ascertain a reason for Drummond and Cannan's failure to confirm the results of their predecessors one is struck by the fact that they employed *dried* anterior lobe tissue in their feeding experiments, while others have employed *fresh* tissue. Having regard to the susceptibility of tethelin to oxidation, upon which Drummond and Cannan have rightly laid so much emphasis, this procedure was hardly calculated to display its effects.

In the drying of pituitary tissue prior to its extraction with alcohol during the preparation of tethelin, the tissue is mixed with a large bulk of anhydrous calcium and sodium sulphates, whereby the period of drying is abbreviated and the tissue is covered and protected to a considerable extent from the access of oxygen. This procedure is naturally impossible when the tissue is to be administered to animals. When the unprotected tissue is dried with free access to air intense discoloration occurs and it may be conjectured that the greater part of the tethelin is destroyed.

Notwithstanding the inadequate character of their own technique, Drummond and Cannan have not hesitated to make indefensible assertions concerning the work which their experiments are designed to invalidate. In view of the controversial methods which they adopt it is curious to find that they accuse the author of "biassed interpretation," for the following reasons.

In my first experiments upon the growth of mice a batch of 72 animals (36 males and 36 females) were taken from the breeding stock without selection[2] and set aside to serve as normal controls. Fresh batches of animals were taken from the same stock, as rapidly as the available numbers permitted, to serve as the subjects of experimental additions to the diet. These constituted, of course, so many separate experiments for which the one group of animals served as controls.

Now the variability of mice at four or five weeks of age is over 20 %[3], which means that one animal in three may be expected to deviate by over 20 % from the average. This being the case, batches of 36 or 24 animals of

[1] The mothers of these animals were fed with pituitary tissue until the young were weaned (at three weeks). But the investigations of Robertson and Delprat [1917] have shown that administration of tethelin to the mother is without effect upon the growth of suckling young.

[2] Excepting that involved in the rejection of animals which were obviously sickly.

[3] Computed as the ratio of the standard deviation to the average weight.

the same sex, chosen without selection at this age, could not possibly be expected to display equal average weights. To attain such an end would necessitate either selection of individuals chosen to display equal averages, a procedure of very questionable theoretical validity, or else the employment of impossibly huge numbers. Each experimental group, therefore, deviated initially in some degree from the normal group and these deviations were sometimes in a negative sense and sometimes in a positive[1].

This being the case, Drummond and Cannan proceed as follows: "For example it is significant that, in his experiments the effect of feeding egg-lecithin to batches of normal mice chosen at random at the age of four weeks showed a difference of 1·5 g. in mean weight, *i.e.* 13 % of body weight—a difference of the same order as the defection of weight of tethelin-fed mice at the maximum divergence of this group from the normal. Yet this, in the former case, is explained away as being due merely to the high variability at that age, whilst in the latter case it is reported as being a marked defection due to the effect of tethelin."

The maximum divergence to which Drummond and Cannan refer occurred at 15 weeks of age, when the variability had fallen to 9 %. The extreme variates had approached one another as they always do during growth [Robertson, 1916, 1; Thompson, 1917] and the animals by now represented a much more uniform type. They differed from the normals by an amount equal to no less than seven times the probable error of the difference computed from the variabilities of the normal and tethelin-fed groups. Yet, according to Drummond and Cannan, this means no more than a deviation of three times the probable error, observed in a different group of animals! To anybody acquainted with the elements of statistical analysis, however, a deviation of seven times the probable error is 19,000 times as significant as a deviation of three times the probable error, since in the first case there is but one chance in 420,000 that the observed deviation could have been accidental, while in the second case there is one chance in 22.

However, let us accept Drummond and Cannan's strange conclusion and suppose that the initial differences of highly variable young are continued in the same proportion in slightly variable animals of more advanced age. It then becomes of crucial importance to know what was the initial deviation of the *tethelin-fed* group from the normals. By a strange oversight Drummond and Cannan omit to mention this. The initial deviation of this experimental group (males) from the average of the normals, as stated in my tables, was + 0·60 g. (1·3 times the probable error of the difference), or $4\frac{1}{2}$ % of the mean weight at that age *in the opposite direction to the maximum divergence from the normal which was subsequently displayed.* How are we to interpret this? On

[1] The consequences of the extreme deviation noted in the lecithin-fed class, to which Drummond and Cannan refer, are carefully considered in our communication of 1919 under the headings "Effects of Egg Lecithin" and "Possible errors of sampling" [Robertson and Ray, 1919].

Drummond and Cannan's own showing the initial divergence of the tethelin-fed group from the normal actually emphasises the significance of the subsequent outcome, and yet they forbear from mentioning it.

The objection is urged that the ages of the mice employed were not identical, since some mice were three days in advance of or behind the others. This arises from my procedure in taking, for example, mice of from 25 to 31 days of age inclusive as having been weighed on the 28th day. This bracketing is employed throughout for obvious technical reasons. The maximum divergence of age is three days, an important item without doubt at three or four weeks of age. But will Drummond and Cannan seriously maintain that it is important at 15 weeks of age?

It is stated by Drummond and Cannan that many other examples of "biassed interpretation" occur throughout my papers, but one further example will suffice. "In support of his contention that mice fed upon pituitary are, size for size, heavier than those normally fed, Robertson reproduces a photograph of two mice of the same age and linear dimensions, one a normal mouse weighing 30 g., the other a pituitary-fed mouse weighing 37 g. When it is pointed out that the weight of the normal was about the mean for his batch, whereas the other weighed 27 % more than the mean for his batch—*i.e.* was an extravagantly abnormal member—the fallacy of the argument is apparent."

How shall I satisfy these critics? My contention was that the condition of mice fed for a prolonged period upon pituitary tissue resembled that of acromegaly in that size-for-size, their weights were in excess of normal, while weight-for-weight they were smaller than normal. My photograph illustrated this difference of "build." What alternative photograph should have been taken? If I had taken a pituitary-fed mouse of average weight and displayed it beside a normal mouse of the same weight, then this would necessarily have been an "extravagantly abnormal member" of the control group. If I had displayed two mice resembling each other neither in weight nor size, the comparison would have been meaningless. If I had been able to exhibit mice from each group resembling one another both in size and in weight, the effect to which I drew attention would not have existed. It is clear that no means of comparison could have been so devised as to meet with the approval of Drummond and Cannan, but that by their criticism they have unintentionally contributed eloquent testimony to the alteration of bodily proportion which was induced in my mice by pituitary feeding.

Conclusions.

1. The experiments of Drummond and Cannan upon the composition and properties of tethelin are, in part, repetition of experiments previously performed by the author and, in part, vitiated by a faulty method of preparation.

2. The experiments of Drummond and Cannan upon the administration of anterior lobe tissue to mice afford no evidence either for or against the view that tethelin contains a growth-controlling constituent of the pituitary body.

3. The statistical method of comparing the growth of normal animals with that of animals in receipt of dietary additions must of necessity be superior to the haphazard method of comparing individual growth-curves or curves constructed from the growth of a few animals. It is, in fact, the only method properly applicable to this type of problem. The criticisms directed by Drummond and Cannan against the author's employment of this method are based in part upon misstatements of fact and in part upon misapprehension of the method.

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XI. A NOTE ON THE HYDROLYSIS OF PECTIN.

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(*Received January 9th, 1923.*)

In a communication to this Journal [1921] the present author showed that when pectin was treated with cold, aqueous alkalis or with the enzyme, pectase, pectic acid was formed, together with methyl alcohol and acetone. It was stated, moreover, that "the proportion of acetone to methyl alcohol produced appeared to be about one part of the former to two of the latter," and, furthermore, that "it appears likely, therefore that pectin is the dimethyl-*iso*-propenyl ester of pectic acid." This conclusion was not based on any indirect method of estimation of either the methyl alcohol or the acetone, which cannot easily be determined accurately in the presence of one another, but was a considered conclusion based on the actual amounts of these substances obtained after their separation by a prolonged and careful fractional distillation and subsequent treatment of the "acetone fraction" with calcium chloride. The amount of material dealt with was large, and more than 3 g. of practically pure acetone were obtained. The author sees no reason to modify his original, somewhat guarded statement.

In view, however, of several personal communications from an authoritative American source the subject appears to require a little further explanation.

The author's statement regarding the formation of acetone on cold[1] hydrolysis was at first denied, but has been subsequently confirmed, here and in America, but it must be pointed out that too prolonged treatment of the pectin with water at a high temperature, especially in the presence of acid, results in a loss of both methyl alcohol and acetone. It has now been stated, however, that whether the percentage of methyl alcohol yielded by pectin is calculated from the amount of alkali required to hydrolyse the latter, or estimated by Zeisel's method, practically identical results are obtained. From this it is concluded that there is hardly room for an *iso*-propenyl group in the molecule. It will at once be seen, however, that the amount of alkali required to eliminate an *iso*-propenyl group is the same as that required for a methyl group. Moreover, when employing Zeisel's method, acetone (or an *iso*-propenyl group) is converted into *iso*-propyl iodide, which readily volatilises and yields the same amount of silver iodide as would a methyl group.

This behaviour of acetone does not seem to be generally realised, and it is considered advisable to draw attention to it as several previous workers have employed Zeisel's method for estimating the methyl alcohol yielded by pectin.

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[1] Acetone is yielded by a great variety of products on treatment with hot alkali.

XII. UREASE. PART I. THE CHEMICAL CHANGES INVOLVED IN THE ZYMOLYSIS OF UREA.

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Urea in aqueous solution is readily converted into ammonia and carbon dioxide by a definite class of ferments. The chemistry of this change attracted attention as far back as the end of the eighteenth century, when Fourcroy and Vauquelin (1798) proved that when urine undergoes alkaline fermentation on exposure to the air the ammonia formed is produced from the urea originally present in the urine.

Pasteur [1861] showed that a living organism was responsible for the alkaline fermentation of urine. The organism was isolated three years later by van Tieghem [1864], and was given the name *Micrococcus ureae*. Musculus [1874, 1876] obtained an enzyme from putrid urine, which he found capable of decomposing urea in aqueous solutions. Lea [1885] cultivated the *Micrococcus ureae* in quantity, and confirmed the results of Musculus.

Over 30 different micro-organisms capable of decomposing urea were recognised and described by Miquel [1890], who first suggested the name *urease* for the enzyme common to all these ureaclastic organisms. Since the discovery by Takeuchi [1909] of soy-bean urease, a plentiful supply of the enzyme has been assured, and much attention has been devoted to the study of the decomposition of urea by urease during the intervening years. Results of great practical value in connection with the estimation of urea have been obtained, but the actual chemical mechanism of the change does not seem to have been investigated very closely since Dumas [1830] suggested the equation

$$CON_2H_4 + 2H_2O = (NH_4)_2\,CO_3$$

to represent the direct hydrolysis of urea in aqueous solution.

The zymolysis of urea has very generally been taken without question to be a simple direct hydration of the urea molecule, either free in solution or combined with the enzyme.

Armstrong and Horton [1912] considered the possibility of urea being in solution in the hypothetical hydrated form of "carbamide," $C(OH)_2(NH_2)_2$, and producing on hydrolysis ortho-carbonic acid and ammonia.

Yamasaki [1920], from the reaction rates and temperature coefficients of the zymolysis, maintains that the decomposition of urea takes place in two definite stages, with the formation of ammonium carbamate as the inter-

mediate product. However, neither of the suggestions affects the general assumption that urea is broken down in solution by a direct hydration process.

If this be so, it seems rather remarkable that the attempts to obtain urea by a reverse synthesis from ammonium carbonate and carbamate, have been so unconvincing, more especially when it is considered that urea is a much more stable compound than either ammonium carbonate or carbamate.

Barendrecht [1919, 1921] has put forward a "radiation" theory of enzyme action. He asserts that when the urease "radiation" strikes a molecule of urea it is absorbed, and as a result the urea is hydrolysed. No explanation is offered as to how this hydrolysis takes place. In support of the "radiation" theory, Barendrecht claims to have observed a reverse zymolysis in solutions where the urease is "decaying," and to have obtained a synthesis of urea from ammonium carbonate solutions and urease "decaying" through the combined influences of alkalinity, time, and temperature.

The validity of Barendrecht's claim is questioned by Mattaar [1920], who asserts that small quantities of urea when added to solutions of ammonium carbonate are destroyed by urease under conditions comparable with Barendrecht's alleged synthesis.

When it is realised that such a synthesis of urea is based on an entirely erroneous conception of the relationship between urea and ammonium carbonate, it will be seen that its occurrence is open to doubt.

The decomposition of urea in aqueous solution.

It was first proved by Fawsitt [1902] that the hydrolysis of urea by acids, alkalies, or water alone at the temperature of 100° does not proceed along the lines of the familiar equation suggested by Dumas in 1830. An intermediate cyanate stage was demonstrated, and Fawsitt concluded that urea in solution is first converted by an intramolecular change into ammonium cyanate, and that the ammonium cyanate is hydrolysed to ammonium carbonate. The influence of acids, alkalies, and alcohol on the change were also investigated [Burrows and Fawsitt, 1914].

E. A. Werner, in the course of his extensive work on the constitution of urea, has discussed these experiments in detail [1918], and has shown that the so-called "hydrolysis" of urea under all conditions consists of two stages: (*a*) the simple dissociation of the urea molecule into ammonia and free cyanic acid, and (*b*) the hydrolysis of the cyanic acid by the solvent [1913, 1918, 1920].

$$(a)\quad CON_2H_4 \rightarrow NH_3 + HNCO,$$

$$(b)\quad HNCO + H_2O = NH_3 + CO_2.$$

It has, of course, long been known that cyanic acid is formed when aqueous solutions of urea are heated above 70°, and advantage of this fact has been taken by Walker and Hambly [1895] to prepare pure silver cyanate, but the significance of this formation of cyanic acid has only recently been realised.

Up to the present, the Werner formula for urea has not been considered in connection with the enzyme decomposition of urea. If urease acts in the manner generally ascribed to catalysts and brings about zymolysis by accelerating greatly a normal chemical transformation, it seems probable that the mechanism of the decomposition of urea by urease is comparable with the decomposition of urea by acids, and by alkalies.

If this be so, a careful chemical examination of the sytem during zymolysis should reveal the presence of the products of dissociation.

The urea/urease system.

In studying an enzyme system attention must be devoted to the following aspects: (1) the enzyme preparation, (2) the application of the enzyme, (3) the substrate, (4) the methods of analysis.

(1) *The enzyme.* A very satisfactory preparation of urease is obtained by extracting the fresh finely ground meal of soy-bean with ether in a Soxhlet extractor for six to twelve hours. By this means most of the fatty material is removed and a very active uniform dispersion is obtained by shaking up with water or with weak alcohol [Folin, 1919]. A better preparation may be got by extracting with absolute alcohol immediately after the ether treatment, but for the present work, attention was devoted to obtaining a standard preparation of uniform zymolytic properties rather than an enzyme preparation of approximate purity.

(2) *The application of the enzyme.* In a qualitative study of enzyme-action it is convenient to have some means of separating the enzyme from the main bulk of the substrate solution so that the subsequent analysis will not be complicated by the presence of enzyme material and debris. This was accomplished by enclosing the enzyme in a collodion tube in one group of the experiments, so that although the substrate had free access to the enzyme the latter did not escape into the bulk of the solution.

Several types of collodion tubes were examined. The most satisfactory ones were made by the Hatschek formula [1920]. The tubes were tested by dialysing urease solutions against distilled water and solutions of ammonium carbonate. Tubes that allowed sufficient enzyme material to escape in 24 hours at room temperature to give a positive biuret reaction on testing the surrounding fluid were rejected.

These collodion tubes are very permeable towards urea and ammonium salts.

In employing collodion separators in enzyme research it is necessary to consider the possible inactivation of the enzyme.

Urease suspensions of the strength used in the experiments were not found to be seriously affected by being kept in the tubes for periods up to two days. After that time, inactivation was apparent in the weaker suspensions, although this seemed to be not so much due to the loss of a dialysable component, or

"co-enzyme," as claimed by Onodera [1915], but rather to be an instance of membrane adsorption of the enzyme.

To obtain a large surface of enzyme suspension exposed to the substrate, a test-tube of smaller diameter was inserted into each collodion tube so that the urease occupied the space between the two tubes. Inverted specimen jars of capacity 100 to 1000 cc. with narrow necks were used to contain the substrate. The collodion tubes were of such size that they almost filled the necks of the jars.

The hydrogen ion concentration of the system was kept within the working range of the enzyme (*i.e.* p_H 6·8–7·2) by means of centinormal nitric acid and the use of a Cole-Onslow comparator.

(3) *The substrate.* The urea used was purified by two crystallisations from water, and was free from all cyanate, carbonate, carbamate, and ammonia reactions. Solutions of urea in water are readily attacked by the micro-organisms of the ordinary laboratory air, and, consequently, the substrate solutions were made up with sterile water and kept in clean stoppered bottles.

(4) *Methods of analysis.* The analysis of the products of urea decomposition in simple solutions is not a difficult matter, but when the solutions are liable to contain materials extracted from the enzyme special precautions have to be taken. Ammonia was detected by the usual Nessler method, and was estimated by aspiration and Nesslerisation or titration in a comparator.

The presence of traces of ammonia in the urease was of no consequence in most of the experiments. When necessary, it can either be estimated and allowed for, or removed by a few days extraction of the enzyme by absolute alcohol at 30°. Folin [1919] has advocated the use of "permutite" but the common fuller's earth seems to be effective also.

It may be noted, in passing, that solutions of urease will develop ammonia when kept for some hours in contact with strong alkalies at room temperatures.

Carbon dioxide was detected and estimated by aspiration into standard barium hydroxide solutions.

Cyanic acid was detected and isolated as the silver salt, by which means it has been found possible to separate it from carbonates and carbamates.

This separation is effected by adjusting the hydrogen ion concentration of the solution to p_H 5·0 by means of $N/50$ HNO_3, and a comparator, using methyl-red as indicator. Under these conditions, all the silver carbonate (and carbamate, if any) is dissolved, and silver cyanate remains.

The presence of the cyanate radicle in the silver precipitate may be demonstrated by three colour tests.

For detecting small traces of cyanate the following method was devised. The silver precipitate, separated from carbonate and carbamate, is collected on a small filter paper and washed thoroughly with distilled water until the washings no longer give any ammonia reaction with Nessler's reagent. The precipitate on the paper is treated with a few drops of warm $N/2$ HCl; this liberates and hydrolyses any cyanic acid present, and on washing the

precipitate once more, the filtrate gives a positive Nessler reaction. This is an exceedingly delicate test for cyanates, provided proper precautions are taken to see that the reagents are ammonia-free. The cyanate radicle may also be demonstrated by treating the precipitate with a few drops of 5 % hydroxylamine sulphate, and after a couple of minutes adding a little dilute ferric chloride; a purple colour is produced. This test is due to Hantzsch and Sauer [1897]. The colour is due to the liberation of cyanic acid and the combination of this with hydroxylamine to form a hydroxy-urea, the ferric salt of which is dark purple in solution. It has been developed by the present author into a method for estimating cyanates colorimetrically. Certain precautions must be taken in applying the test to traces of cyanates. The presence of much ammonia interferes with the test by obscuring the characteristic black-purple shade. The hydroxylamine must remain in contact with the free cyanic acid for sufficient time to interact to form the hydroxy-urea. In the presence of excess of the hydroxylamine the coloration is not permanent as the ferric salt is soon reduced to an almost colourless ferrous salt. The colour can be restored by careful addition of more ferric chloride or by adding a drop of hydrogen peroxide solution (which must be free from acetanilide, or any other preservative which gives a colour with ferric salts). Once the solution has been freed from uncombined hydroxylamine the colour is permanent.

Cyanates formed in solution may also be identified by the fact that the free cyanic acid combines with urea to form biuret.

Cyanates in quantity can be most rapidly shown (as, for example, the sublimate formed by heating dry urea in a test-tube) by the intense blue colour given with solutions of cobalt acetate or nitrate.

The delicacy of these tests under general conditions is as follows. Nessler method, 1 in 400,000; hydroxylamine reaction, 1 in 2000; cobalt method 1 in 90; but the delicacy can be greatly increased by using the centrifuge to collect the silver precipitates and by careful washing with ice-cold water.

Biuret was identified and estimated colorimetrically by the copper method, as described by Werner [1913], under conditions when the absence of foreign protein material derived from the enzyme was assured.

These analytical methods are grouped here for convenience, as they will be referred to in subsequent papers.

Experimental. Series I. Detection of cyanates in the urea/urease system.

Fresh solutions of pure urea in distilled water saturated with CO_2 were prepared of concentrations 1, 5, 10, 20 %, and submitted to the action of freshly extracted urease (1 g. in each collodion tube). The solutions were kept at low temperature, 1°–5°, to retard hydrolysis of the cyanic acid. At hourly intervals samples were drawn off from the outer containing jars, treated with $N/10$ $AgNO_3$, the solution set to p_H 5 by $N/50$ HNO_3, and filtered through a close filter paper.

After two hours all the solutions gave positive reactions for cyanic acid by the Nessler method, this continued for several hours in the solutions, but entirely disappeared from the 1 % solution on keeping the flasks at room temperature overnight. All the solutions next day gave a definite biuret reaction, most marked in the strongest solution of urea. By means of a control it was found that this did not arise from the escape of protein material from the dialysing tubes, but was due to the interaction between cyanic acid and the substrate.

Cyanic acid was also shown in solutions of urea undergoing zymolysis in ordinary flasks in direct contact with the enzyme, by treating samples drawn off from time to time with excess of colloidal ferric hydroxide, which prepipitates nearly all the ammonia from the solution and brings down the enzyme with the precipitate. On centrifuging the samples, cyanates were found to be present in the clear supernatant layer by applying the hydroxylamine test, and by pipetting off the clear solution and applying the silver method.

The conclusion from a large number of experiments of this type is that cyanic acid is produced during the decomposition of urea by urease. The yield of cyanic acid as silver cyanate in these experiments was always small; up to 0·1 g. from a litre of 5 % urea solution being obtained.

Experimental. Series II. Estimation of the cyanic acid formed during the zymolysis of urea.

Method A. Substrate, 5 % urea. Enzyme, 5 % extracted urease suspension which had been kept for three days at 40°, until the coarser particles had subsided. Samples of 20 cc. of substrate and 10 cc. of enzyme suspension were mixed at 40°, set to p_H 6·5, and incubated at 40°.

At hourly intervals, duplicate pairs of flasks were withdrawn and each treated with 1 cc. of $N/1$ $AgNO_3$. One flask was then titrated to p_H 5 with 0·6 N HNO_3, using methyl-red paper[1] and a standard flask for comparison.

The acid used gave a measure of the relative degree of zymolysis, and also dissolved any silver carbonate or carbamate that might be present, leaving silver cyanate, silver chloride and precipitated enzyme.

The contents of the other flask were then treated with the same amount of 0·6 N HNO_3, to bring it to p_H 5 using no indicator. Both samples were then filtered and the precipitates washed three times with a fine jet of cold distilled water. The silver in the filtrate was determined by titration with thiocyanate of such strength that 1 cc. corresponded to 0·015 g. of potassium cyanate. The difference between these values and the value of the silver originally added represented the silver left on the filter paper. By titration, the amount of silver nitrate combining with 10 cc. of the uniform enzyme suspension was determined under comparable conditions, and the difference

[1] Methyl-red paper was used in preference to the solution, so as to have a colourless liquid for the subsequent titration.

between the amount of silver required to precipitate the enzyme together with associated inorganic salts, and the amount of silver left on the filter paper gave the silver fixed as silver cyanate. The presence of cyanic acid in the silver residue was confirmed by the tests described.

The enzyme suspension employed was sufficiently uniform to give an almost constant silver value in a series of six controls.

The chief error was found to lie in the washing of the precipitate on the filter paper. If it is washed for some time with distilled water at room temperature the silver gets ultimately washed away from its union with the protein, but by adopting a uniform technique this error was rendered negligible.

Table I.

Time in hours	Percentage urea decomposed	Percentage cyanic acid present
0	0	0
1	11·16	Not estimated
2	24·84	0·007
3	30·78	0·009
4	32·28	0·011
5	35·98	0·010
7	45·00	0·010

Temperature, 40°.
Enzyme, 10 cc. uniform suspension of urease decanted from 5 % suspension.
Substrate, 20 cc. 5 % urea.
Initial p_H 5·5.

Here it will be seen that the concentration of cyanic acid rises to a maximum and remains remarkably constant. The concentration will obviously depend on the difference between the rate of formation and the rate of removal by hydrolysis of the cyanic acid. When this difference in the rates is constant the concentration of cyanic acid will be constant. Since cyanic acid is very readily hydrolysed in weak aqueous solution at room temperature, it might be expected that the lower the temperature of the urea solution undergoing zymolysis the higher would be the concentration of cyanic acid, since it would accumulate in the solution. A series of experiments showed that this is not so. The amount of cyanic acid in solution is a function of the rate at which the urea is being decomposed, and this decomposition has a higher temperature coefficient than has the decomposition of the cyanic acid by hydrolysis.

To reduce as far as possible the errors introduced into the estimation by the presence of excess of urease material a method was devised whereby the cyanic acid could be estimated in solutions free, or almost free, from enzyme.

Method B. Estimation of cyanic acid. The urease was enclosed in a collodion sac and the cyanic acid was estimated in samples drawn from the surrounding solution. The proportions were 10 cc. of 10 % urease solution in the sac to every 100 cc. of 2 % urea solution in the containing vessel. At definite intervals 20 cc. samples were drawn off in duplicate, set to p_H 5·0 using 0·7 *N* HNO_3, and methyl-red paper. One sample was boiled for half a minute to destroy any cyanic acid present, then each sample was treated with an excess of

$N/10$ $AgNO_3$, filtered, and the silver estimated in the filtrate. The difference in the silver value gave the amount of cyanate present. The substrate and the enzyme in these experiments were in the ratio of 100 cc. 2 % urea to 10 cc. 2 % urease.

Table II.

Temperature of experiment	Maximum percentage of cyanic acid
2–3°	0·0016 after 45 hours
5°	0·0043 „ 30 „
7·5°	0·0034 „ 20 „
10°	0·0301 „ 24 „

At temperatures above 10° the collodion sacs became very fragile. In general, the lower the temperature the longer the cyanic acid took to attain its maximum value. In the experiments carried out at a temperature just above freezing point, the cyanic acid attained a concentration of 1·6 mg. per 100 cc. substrate when 5·01 % of the total urea had been decomposed. On leaving the solution at 5° for another period of 15 hours the concentration of the cyanic acid had fallen to 0·3 mg. per 100 cc. owing to its slow hydrolysis in the alkaline liquid.

Significance of the presence of cyanic acid in the urea/urease system.

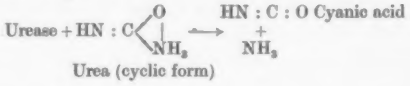
Cyanic acid may represent the intermediate stage in the zymolysis of urea, or may be the result of some comparatively unimportant side reaction. Since the acid is not formed from sterile solutions of urea at the temperature of the experiments, it must be produced by the action of the enzyme on the urea (urease alone, of course, contains no cyanates nor gives rise to them in simple solution). From what is known of the part played by this substance in the normal decomposition of urea, it is probable that it is the true intermediate compound in the enzyme decomposition of urea.

Up to the present, Yamasaki [1918, 1920] appears to be the only worker who claims to have detected ammonium carbamate in the urea/urease system. Unfortunately, his results can hardly be considered conclusive since the method he adopts for estimating carbamate is equally applicable to cyanate. Yamasaki, working on the basis of the old "carbamide" formula for urea, does not appear to have considered cyanic acid at all in connection with the change, which he ascribes to a successive hydrolysis of the urea by simple catalytic action in the substrate—an explanation that affords no light on the mechanism of the process.

By the methods described in the present paper, cyanic acid can be detected and isolated as the silver salt in the presence or absence of carbamates, and, while there is no experimental evidence that ammonium carbamate is not formed during the enzyme decomposition of urea, there is evidence that cyanate does occur and that the decomposition of urea by urease proceeds along the same chemical lines as the normal decomposition of urea in solution by acids, alkalies, or the action of heat.

The change may be represented as follows:

(*a*) *Dissociation stage:*



(*b*) *Hydrolysis stage:*

$$HN : C : O + H_2O \longrightarrow NH_3 + CO_2$$

Cyanic acid

Urease is not directly concerned in the "hydrolysis" of urea. The function of the enzyme is to bring about dissociation of the urea into ammonia and cyanic acid. The hydrolysis of the cyanic acid follows as a secondary change in the presence of water. This has been supported by observations on the action of urease on solutions of urea in alcohol, where the cyanic acid could not undergo hydrolysis.

It will be shown that this conception of urease as a *dissociating* enzyme and not a *hydrolysing* enzyme provides a new light for the investigation of enzyme mechanism, and accounts for, amongst other things, the specific action of the ferment.

Conclusions.

1. Cyanic acid has been isolated as the silver salt from solutions of urea undergoing decomposition by urease.

2. Cyanic acid attains to a maximum concentration in the urea/urease system, and is being continually produced as fast as it is removed by the hydrolytic action of the solvent.

3. Cyanic acid and ammonia are the intermediate products in the enzyme decomposition of urea.

4. The enzyme decomposition of urea proceeds along the same lines as the normal decomposition of urea in solution by acids, alkalies, or heat.

5. Urease is a *dissociating* enzyme; decomposing the neutral urea molecule into an alkaline component, ammonia, and an acid component, cyanic acid, which is rapidly hydrolysed by the solvent into ammonia and carbon dioxide.

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XIII. THE STEROL CONTENT OF COW'S MILK.

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The following account of the nature and composition of the so-called "unsaponifiable matter" found in milk fat, originated from analyses required for some experiments on cholesterol metabolism in adults. Ordinary cow's milk contains nearly one-tenth per cent. of a mixture of substances which can be extracted with ether or light petroleum after saponification of the fat by alkalis, and after evaporation of the ether is obtained as a soft golden yellow solid. In spite of the large number of investigations that have been made upon milk fat, this fraction has been invariably dismissed, until recently, by saying that it consisted of traces of cholesterol, wax, colouring matter etc.; this was no doubt largely due to the lack of any good method of separation or estimation of the constituents.

In 1883, Schmidt-Mülheim [1883] drew attention to a substance present in milk which he considered to be cholesterol on account of its colour reaction. Menozzi [1903] was perhaps the first to investigate the substance more closely and he showed that the sterol was identical in physical and chemical properties with the cholesterol isolated from bile. The earlier quantitative estimations of the cholesterol content of milk were made by Magnus-Levy [1910], Tolmatscheff [1867], Siegfeld [1906], Grigaut [1913], and others. Their values range from about 0·01 to 0·04 g. per 100 cc. milk.

In 1918 Denis and Minot [1918] published a series of analyses both of cow's and human milk made by Bloor's colorimetric method [1916]; this method however is known to give rather too high results for some tissues. Their figures for the fat and total cholesterol in 100 cc. of cow's milk are given in Table I.

Table I.

Fat	5·0	4·6	4·4	4·2	4·7	3·4	5·3	3·4
Cholesterol	0·0176	0·0176	0·0164	0·0162	0·0158	0·0156	0·0152	0·0152
Fat	4·0	3·9	3·3	4·5	3·7	3·4	3·2	
Cholesterol	0·0144	0·0140	0·0136	0·0128	0·012	0·0112	0·0105	

Average fat, 4·07. Average cholesterol, 0·0145.

The average percentage of cholesterol per 100 g. of fat is thus 0·356. Last year, while our work was in progress, Wacker and Beck [1921] published a series of determinations made by the digitonin method of Windaus, which are summarised in Table II.

Table II.

Fat in 100 cc. milk	4·02	4·00	3·75°	3·72	3·69	3·46	3·31	3·31
Cholesterol in 100 cc. milk	0·014	0·014	0·016	0·012	0·011	0·012	0·011	0·011
Cholesterol in 100 g. fat	0·351	0·352	0·424	0·315	0·300	0·347	0·329	0·327

Averages: 3·65; 0·0126; 0·343.

The work to be described in the present paper may for convenience be divided into three parts: (1) quantitative estimation of cholesterol content of fresh and condensed milk; (2) qualitative examination of the fraction of the sterols precipitable by digitonin; (3) separation and quantitative examination of the residual non-precipitable oils.

Method of analysis.

All our analyses have been made on the fat as separated by the well-known Gottlieb-Rose method.

The fat was divided into two portions, one for determination of the free and the other for determination of the total free and combined cholesterol. The estimation was made by the method described by Fraser and Gardner [1910] with some slight modifications, which we detail briefly. To estimate the total free and combined cholesterol the fat was dissolved in ether and a large excess of a solution of sodium in 97 % pure alcohol, containing about 8 to 10 times the theoretical quantity of sodium required, added. This mixture was allowed to stand 24 hours, and then heated under a reflux on the water bath for 4 to 5 hours, in order to ensure the complete hydrolysis of cholesterol esters. It was then largely diluted with water and repeatedly extracted with ether until nothing more was dissolved. The ethereal solutions were united, thoroughly washed by shaking with water until quite free from traces of soap and made up to known volume. A suitable aliquot portion was then evaporated until free from ether, dissolved in alcohol and precipitated with a considerable excess of a 1 % alcoholic solution of digitonin. After standing overnight the alcohol was completely evaporated at the lowest convenient temperature. The residue was washed by decantation with ether to remove unprecipitated oily matter, then with warm water to remove excess digitonin. Finally the precipitate of cholesterol-digitonide was brought on to the Gooch, the washing completed and the substance dried and weighed.

For estimation of free cholesterol the fat was dissolved in alcohol and precipitated directly with excess of 1 % digitonin solution. In this case it was found advantageous not to evaporate the alcohol completely, but to leave

about one-fourth of the total volume, otherwise it is sometimes difficult to wash away all traces of fat and colouring matter with ether. After filtering off the alcohol and washing the precipitate with ether the rest of the procedure was as described above.

Fresh milk.

For fresh milk we used two samples of "Grade A certified milk," as defined by the milk section of the Ministry of Health, and two samples of so-called "Nursery Milk" stated to be obtained from a herd of tuberculin tested cattle which yield a milk higher than the average in butter-fat. The results are given in Table III.

Table III.

No.	Description	Fat per 100 cc. g.	Unsaponifiable matter in 100 cc. milk g.	Free cholesterol per 100 cc. g.	Ester cholesterol per 100 cc. g.	Total cholesterol per 100 cc. g.	Total cholesterol in 100 grams of fat g.
(I)	Nursery milk (1922)	4·46	—	0·001	0·015	0·016	0·358
(II)	" " "	4·14	0·0725	0·0144	0·0029	0·0173	0·418
(III)	Grade A (1922)	3·38	0·082	0·0114	nil	0·0114	0·334
(IV)	" "	3·47	0·066	0·0113	0·0044	0·0157	0·344
	Mean	3·86	0·0735	0·0095	0·0056	0·0151	0·3635

Condensed milk.

In order to cover a wider range of conditions, we also examined a number of samples of condensed milk. These were ordinary shop samples, described as consisting of whole milk, and had been prepared in England, United States, Norway, Denmark and Switzerland. The results are given in Table IV.

Table IV. *Condensed milk, bought* 1920 *and* 1921.

Description	Specific gravity	Total solids per 100 g. milk	Ash per 100 g. milk	Protein total N ×6·38 per 100 g.	Fat	Total unsaponifiable matter per 100 g. milk	Total cholesterol free and ester per 100 g. milk	Total cholesterol per 100 g. milk fat	Unsaponifiable matter not precipitated by digitonin per 100 g. milk fat	Remarks
(1) Unsweetened	1·085	28·12	1·43	7·44	8·47	0·118	0·0336	0·401	1·000	same makers
(2) Sweetened (*a*)	1·284	80·52	2·14	9·77	10·50	0·133	0·0268	0·255	1·010	same makers
" (*b*)	—	—	—	—	—	0·145	0·0227	0·216	1·060	same makers
(3) Unsweetened	1·074	25·80	1·38	7·12	7·98	0·062	0·0227	0·256	0·529	
(4) "	1·091	32·68	1·63	8·38	10·12	0·067	0·0317	0·314	0·329	
(5) Sweetened	1·294	74·45	1·60	7·70	9·32	0·082	0·0310	0·333	0·542	
(6) Unsweetened	1·072	25·77	1·32	6·61	8·34	0·125	0·0428	0·513	0·990	same makers
(7) Sweetened	1·309	75·58	1·70	7·33	8·65	0·161	0·0486	0·562	1·298	same makers
(8) "	1·288	73·20	1·78	7·60	9·30	0·053	0·0248	0·256	0·314	
(9) Unsweetened	1·073	26·40	1·47	7·05	8·22	0·044	0·0201	0·245	0·293	
(10) Sweetened	1·308	76·16	1·81	8·03	8·96	0·055	0·0266	0·295	0·335	
(11) "	1·305	77·23	1·94	8·78	10·89	0·085	0·0317	0·291	0·490	
				Mean	9·16	0·094	0·0303	0·328	0·691	

For the sake of comparison, the average results of various observers are brought together in Table V.

Table V.

	Fat per 100 cc. milk	Cholesterol per 100 cc. milk	Cholesterol per 100 g. milk fat
Denis and Minot	4·07	0·0145	0·356
Wacker and Beck	3·65	0·0126	0·343
Fox and Gardner (fresh milk)	3·86	0·0151	0·364
,, ,, (condensed milk)	—	—	0·339
Mean	3·86	0·0141	0·351

It will be seen from the above tables that the percentages of cholesterol found by the various methods quoted are in fairly close agreement.

Ratio of total cholesterol to fat.

The most obvious preliminary point to settle seems to be whether there is any definite relation between cholesterol and fat content. The average figures in Table V would at first sight indicate that there is a rough proportionality. This has already been pointed out by Denis and Minot, and their table, quoted above, which is arranged in order of descending values of cholesterol is suggestive, although there are several quite marked exceptions. Neither the figures of Wacker and Beck nor of ourselves however point to any exact ratio. It may also be mentioned that no such exact proportionality is found in tissues, blood etc. It will be noticed that the condensed milks show a much greater variation in the percentage of cholesterol in the fat than the specimens of fresh milk examined. Assuming that these variations are real and do not merely result from the removal or addition of cream or from the various processes of manufacture employed, they may perhaps be accounted for by differences of breed, feeding, climate, etc.

Breed. The percentage of fat varies very markedly with the breed of cow. The extent of the variation is well shown by the following figures, compiled by Wing [1897] from a large number of analyses.

Breed:—	Friesian-Holstein	Shorthorn	Ayrshire	Jersey	Guernsey
Average % of fat in milk	3·51	3·65	3·68	4·78	5·02

In actual practice the range may be considerably higher as it is not very uncommon for a Friesian cow to give milk with a fat percentage below 3, and for a Guernsey to reach 6 or even 7 %. In this connection however it must be remembered that the total volume of milk produced also varies and in the opposite sense, so that the actual output of fat in a given period according to Henry [1911] is nearly the same for all dairy breeds.

In order to test the effect of breed on the fat and cholesterol content of the milk we secured two samples, one from a Friesian cow known to yield a milk low in fat and a second from a good average Jersey. The analyses are given in Table VI.

Table VI.

Breed	Fat per 100 cc.	Cholesterol per 100 cc. Free	Ester	Total	Total cholesterol per 100 g. fat
Friesian	2·95	0·0096	0·0023	0·0119	0·404
Jersey	5·96	0·0180	0·0047	0·0227	0·381

Here the output of cholesterol appears to follow approximately the output of fat.

Diet. Of the influence of this factor upon the amount of cholesterol in milk little can be said with certainty. Generally speaking, diet, if sufficient, is stated to have little effect on the milk, either quantitatively or qualitatively. Thus it appears to be easier to increase the yield by constant, regular and complete milking than by increased or richer feeding. What would be the effect of a prolonged period of more or less deficient diet on the fat-cholesterol content of the milk we do not know, but it is perhaps not without significance that three samples of milk, two purchased in London and the other in Cambridge for metabolic experiments in the summer of 1917 and early spring of 1918, gave the following results.

	Fat per 100 cc.	Total cholesterol per 100 cc.	Total cholesterol per 100 g. fat
(1)	3·73	0·007	0·188
(2)	3·50	0·007	0·200
(3)	2·92	0·009	0·308

It is well known that certain feeding stuffs such as cotton and linseed meal have an effect on the physical character of the fat. Further in a series of interesting experiments Palmer and Eckles [1914] were able to show that almost colourless butter can be obtained if the diet is kept sufficiently free from the lipochrome carotene. Here also the shortage is at first made good by reserves in the tissues; this is the probable explanation of "pale" winter butter sometimes met with. Apparently we have here a case of one constituent of milk being directly dependent for its supply upon the diet. Whether the same applies to the sterol there are no data to say, but experiments are in progress which we hope may throw light on this point.

Lactation period. As the lactation advances some changes in the fat content of milk occur, but we do not know whether the sterol follows suit. We have not had an opportunity of examining cow's colostrum from this point of view, but we agree with Wacker and Beck in finding that human colostrum is much richer in cholesterol than normal human milk. This however we propose to deal with in another communication.

Proportion of free cholesterol to cholesterol in form of esters.

According to Wacker and Beck cholesterol is not found in the free state in milk but in the form of esters, presumably esters of oleic and palmitic acids. This conclusion was based on the following experiment. Milk fat was shaken in a separating funnel with hot alcohol. Since free cholesterol is easily soluble

in hot alcohol, while the oleic and palmitic esters are insoluble or difficulty soluble, they expected that any free cholesterol would separate out from the alcohol solution in characteristic crystals, or could be detected by the digitonin reaction. They were unsuccessful in finding free cholesterol by this method. They assumed however without much justification that the presence of fat would not influence the solubility in alcohol. Our own conclusions are almost exactly the opposite. As will be seen from Table III, in all except the first case we found that the main proportion was in the free state. The average ratio of free to ester cholesterol was 1 : 0·54. In the case of the Friesian and Jersey cows (Table VI) the proportion of free to ester cholesterol was in the first case 1 : 0·24 and in the second 1 : 0·26.

Distribution of cholesterol in milk.

Little is known of the manner in which the cholesterol is distributed throughout the milk. Being quite insoluble in water it might be supposed that it was merely dissolved in the globules of fat, but as Wacker has already pointed out this scarcely explains the facts. He finds that the liquid which remains after the fat has been removed either by the separator or during churning is richer in cholesterol than can be accounted for on this hypothesis by the small amount of fat still remaining. According to his analyses the fat in skim milk would contain 0·80 % of cholesterol instead of 0·38. We came to the same conclusion in another way since we noticed that butter fat invariably shows a lower percentage of total cholesterol than the whole milk fat. This difference will be seen from a comparison of the average percentage cholesterol content of milk fat—0·35 in Table IV with the following analyses of samples of genuine butter given in Table VII.

Table VII.

	Total cholesterol %
(1) Reading Agricultural Dept. College Farm herd	0·278
(2) Butter made from milk of Jersey cow ...	0·291
(3) Good commercial sample	0·225
(4) Commercial sample	0·254
Mean	0·244

In order to confirm this we made the following analyses of samples of separated milk and butter milk kindly procured for us by Lord Rayleigh Dairy Co. (Table VIII).

Table VIII.

	Fat per 100 cc.	Cholesterol per 100 cc.			Percentage of total cholesterol in fat
		Free	Ester	Total	
Skim milk	0·187	0·0029	0·0008	0·0037	1·912
Butter milk	0·511	0·0089	0·0012	0·0101	1·977

These facts rather suggest that while no doubt most of the cholesterol—free and ester—is present dissolved in the fat globules, a certain amount may be present in some other form, probably in colloidal solution, or possibly even

combined with the protein. We have found a somewhat similar state of things when examining ascitic fluids, which, even when apparently clear and capable of being filtered through paper without any residue, often contain quite considerable quantities of cholesterol and cholesterol esters.

Qualitative examinations of the sterols of milk.

The sterol which can be precipitated by digitonin, as will be seen from the tables, forms only about one-third to half of the total unsaponifiable matter of the fat, the remainder consisting of sticky yellow oils, very inactive and not easy to deal with or characterise.

In order to examine both precipitable and non-precipitable portions more closely we isolated a considerable quantity, using butter as a convenient source. The bulk of the fat was removed by saponification with caustic soda and the unsaponified portion was subjected to a final saponification by means of sodium ethoxide in alcoholic solution, the mixture being boiled for several hours, in addition to standing 24 hours, in order to ensure decomposition of difficultly hydrolysable sterol esters.

The yield of unsaponifiable matter from butter is somewhat lower than from milk since as mentioned above some sterol remains behind in the butter milk. A further minor source of error, which accentuates this difference between the percentage of unsaponifiable matter in milk fat and butter fat, is due to the small amount of resinification which takes place on hydrolysis by alkalis in alcoholic solution [Gardner and Fox, 1921]. This error is proportionally greater when small quantities of fat are analysed, so that the figures given in the tables for unsaponifiable matter are probably rather too high.

Sterols precipitated by digitonin.

The crude sterol was separated as far as possible from oily matter by crystallisation from acetone or alcohol, and the last traces removed from the oils by precipitation with digitonin. The digitonide was filtered off and decomposed as recommended by Windaus by heating in the vapours of boiling xylene. The whole crystalline matter was now subjected to a thorough fractional crystallisation, melting points taken and the benzoates of several fractions prepared. All the fractions corresponded in properties to cholesterol and cholesterol only. The final residues, too small to purify by further crystallisation, were benzoylated by the pyridine method, but again the result was a typical cholesterol benzoate.

We therefore conclude that the precipitable portion of the unsaponifiable matter of milk fat consists entirely of cholesterol.

Oils not precipitated by digitonin.

We were not successful in isolating any pure substance from these oils, but they appeared to consist in part at least of stable esters which escaped saponification by alkali. We attempted to purify the oils by distillation in

super-heated steam. This helps to remove resinous matter, but also appears to hydrolyse some of the very stable esters which occur in these mixtures. The distillation proceeded very slowly and there was not quite the same tendency for the distillate to assume the characteristic candle-like solid emulsion noted in the case of similar faecal oils [Gardner, 1921]. The distillate was dissolved in ether and the ethereal solution shaken out with alkali. From the alkaline solution a small quantity of a crystalline acid was obtained, which was purified by distillation *in vacuo*. It had no definite melting point and on titration with alkali proved to have an average molecular weight of about 288. The neutral portion after evaporating the ether was distilled in a high vacuum. It passed over between 170–180° for the most part. An attempt was made to fractionate this and eventually two main fractions were obtained: (1) distilling up to 175° (2 mm.) and (2) above 175°. Fraction (1) was a mobile golden yellow liquid at ordinary temperature, but solidified in a freezing mixture. It gave on treatment in chloroform solution with acetic anhydride and a drop of sulphuric acid a dark brown colour which more or less rapidly changed to a dusky olive green very similar to the colour obtained with faecal oils [Gardner, 1921]. On combustion 0·1885 gave 0·2049 H_2O and 0·5629 CO_2. C = 81·44 %, H = 12·08 %.

The fraction 2 was again hydrolysed, this time by heating in a sealed tube at 110° with alcoholic potash. The liquid was diluted and extracted with ether and the ethereal solution shaken out with alkali as before. A very small fraction of acid, apparently a mixture of a liquid and a solid acid was obtained. This was distilled *in vacuo* and by titration with alkali an average molecular weight of about 274 was indicated. The acids were too small in amount for identification, but the liquid portion appeared to be unsaturated.

The neutral portion after removal of the ether distilled in a high vacuum at about 200°. It came over as a clear golden yellow amorphous glass which just melted on a warm day. On freezing out from acetone a trace of a white wax-like substance was obtained too small however for further purification. On combustion of the amorphous glass 0·1543 gave 0·1579 H_2O and 0·4582 CO_2. C = 81·00 %, H = 11·37 %.

On testing an alcoholic solution of this oil with digitonin a mere trace of a digitonide was precipitated, far too small in quantity however to do anything with.

We take this opportunity of thanking Capt. Golding, of University College, Reading, and Miss E. Cornish, of Steyne, for kind help in obtaining some of the samples of milk and butter required, and also the Government Grant Committee of the Royal Society for help in defraying some of the cost of the work.

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XIV. ON THE REDUCTION OF METHYLENE BLUE BY TISSUE EXTRACTS.

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THUNBERG [1909], in the course of an extensive study of the influence of various substances on the gas-exchange of surviving frog's muscle, found that previous treatment of the muscle with succinic acid caused a lowering of the respiratory quotient, in part due to diminished output of carbon dioxide, and, in part, to increased oxygen intake. If, however, the muscle was first subjected to a prolonged washing with water (a procedure which Batelli and Stern [1910] had found to cause a very considerable lowering of the gaseous exchange, the normal exchange being restorable in great measure by the addition of the aqueous extract, due, according to these workers, to the presence in the water-extract of an unisolated substance to which they gave the name "Pnein") and, after this washing, immersed in succinic acid, the gaseous exchange and particularly the consumption of oxygen would be considerably increased. Citric, malic, and fumaric acids were found also to increase the gas-exchange of this water-extracted muscle.

It is now well-known that many animal and vegetable tissues possess the property of reducing methylene blue, a reaction which makes itself apparent in the absence of oxygen by the formation of the colourless form of the pigment. As shown by Harden and Norris [1915] for dried yeast and rabbit muscle, and since then by various workers for many other tissues, this reducing property is removable to a greater or lesser extent by more or less prolonged washing of the tissue with water. Thunberg [1916], influenced by his previous results on the effects on the gas-exchange, showed that the addition of succinic acid to muscle, which had had its power of reducing methylene blue removed to a great extent by thorough washing, would restore its capacity for decolorising the pigment. The reaction was accompanied by the disappearance of the succinic acid and its replacement by fumaric acid, as shown by Einbeck [1913].

In a recent paper, Thunberg [1920] shows that many other substances besides succinic acid may act in a similar manner, and concludes that such

substances are to be considered as intermediary products of metabolism. From these results and the fact that such reactions are not influenced by cyanides, Thunberg concludes that here we have strong evidence in favour of Wieland's hypothesis that the cause of many biological oxidations is to be sought, not in the activation of oxygen but in the activation of hydrogen, the hydrogen being transferred from one substance to another by means of an enzyme. These enzymes have been given by Thunberg the generic name of "Hydrogentransportases" or "Dehydrogenases." In the succinic acid reaction the "Succinodehydrogenase" removes hydrogen from the "Donator," succinic acid, and transfers it to the "Acceptor," in this case, the methylene blue. In living tissues, of course, the acceptor for the removed hydrogen is oxygen, resulting in the formation of water. According to such a conception, the oxygen inspired and transported to the tissues is not concerned in the direct oxidation of the metabolic products, but in the removal of the hydrogen which has been taken from these substances by means of the dehydrogenising enzymes.

The question was raised by Thunberg [1920] as to whether each donator possesses its own specific dehydrogenase, or whether the indirect oxidation of these substances is to be considered as due to the activities of a common enzyme. The results of his experiments on freezing and warming the muscle-mass to various temperatures appeared to indicate that succinic, glutaric, and hydroxyglutaric dehydrogenases, at least, are specific for these substances respectively, a result which is supported by his finding that while *l*-tartaric acid will act as a donator *d*-tartaric acid will not.

The following experiments were conducted primarily with the object of obtaining evidence on the question of the specificity of the enzymes.

In order to obtain uniformity of results, an extract, prepared according to the method described by Ohlsson [1921], by shaking the washed muscle for one hour with a solution of K_2HPO_4 of one-fifteenth molar strength, was employed. 1 cc. of such an extract was measured into a Thunberg vacuum tube, suitable amounts of methylene blue and donator substance added, and a sufficient quantity of water to make the volume in each tube of the experiment the same. The ground stopper, smeared with a mixture of bees'-wax, resin, and vaseline was inserted, the tube evacuated under water with an ordinary water-pump until the contents boiled when warmed with the hand, and then placed in a thermostat at 35°, the time for complete decolorisation of the methylene blue being noted.

Experiments were first conducted to determine which substances would act as donators to such a phosphate extract; only those substances which had been found by Thunberg to act as efficient donators in working with unextracted washed muscle, were tried. All acids were used in the form of carefully neutralised solutions of their potassium salts.

The following table gives the results obtained: (A), with an extract made from ox-muscle; (B), with a rabbit's muscle extract; and (C), with an extract

made from rabbit's liver. The figures given are calculated according to the following formula introduced by Thunberg [1921]:

$$I = 100\left[\frac{1}{A} - \frac{1}{B}\right]$$

(where A is the time required for complete decolorisation in the presence of a donator, and B the time for complete decolorisation when the muscle is acting alone).

The values of I give some idea of the relative efficiency of the various donators. Taking into consideration the possible errors due to leakage and bacterial action, it is doubtful if substances with a value below unity should be considered as hydrogen-donators.

Donator	A	B	C
Succinic acid	14	9	22
Methylsuccinic acid ...	4	3	—
α-Hydroxyglutaric acid ...	2·8	1·3	1·5
α-Ketoglutaric acid ...	2·4	4	2·2
Lactic acid	0·1	2	1·4
Tartronic acid	0·05	1	2
Formic acid	0	0·4	3·4
Acetic acid	0	0·2	0·7
Oxalic acid	See below	0·6	0·7
Glutaric acid	0	0·6	0·5
α-Hydroxybutyric acid ...	0·05	0·5	0
β-Hydroxybutyric acid ...	0·05	0·5	0·7
l-Tartaric acid	0	0·7	—
d-Tartaric acid	0	0·5	—
Glutamic acid	0	0·5	1·1
Propionic acid	0	0	3
Citric acid	0	0	1·4
Alanine	0	0	0·3
Butyric acid	0	0	0
Capronic acid	0	0	0
β-Ketoglutaric acid ...	0	0	0
Dimethylsuccinic acid ...	0	0	—
Methylethylsuccinic acid ...	0	0	—
Diethylsuccinic acid ...	0	0	—

Fumaric acid, malic acid, and maleic acid prevented decolorisation taking place at all in the case of both ox-muscle extract and rabbit-muscle extract, while with oxalic acid the decolorisation time in the case of ox-muscle extract was longer than in the absence of any donator substance.

The first experiments were those performed with extracts made from ordinary butcher-meat, but, as the age of such meat was always doubtful, extracts made from the muscles of a rabbit, killed immediately before the experiment, were afterwards employed. Considerable difficulty was at times experienced in obtaining an efficient extract from rabbit muscle, a difficulty which, sometimes but not always, could be surmounted by grinding the washed muscle with a little powdered glass in a mortar before extraction with the phosphate solution. A too prolonged grinding with glass powder was found to diminish the number of substances which would act as donators; this is in accordance with the observation of Harden and Norris that a too fine division of the muscle leads to the removal of the enzyme. Too pro-

longed washing of the muscle also diminished the number of substances acting as donators. Thunberg's finding that the succinic reaction was by far the most resistant was confirmed.

An attempt was now made to determine whether each donator possessed its own dehydrogenase, *i.e.* whether a number of enzymes, each specific for its own dehydrogenising reaction, were present, by the following method.

Keeping the amount of methylene blue constant, and varying the concentration of donator present, that concentration which would cause complete decolorisation in the shortest time was determined for each of two donators. Then, combining these amounts of donators in the one tube, it was presumed that the time required for complete decolorisation would be less than the shorter of the two times previously obtained if two specific enzymes were in action, while, if there were only one common enzyme, the time would be the same or slightly longer. As succinic, α-hydroxyglutaric, and α-ketoglutaric acids were found to be the most constant donators, these acids were used for such experiments.

The following gives an example of such an experiment carried out with an extract prepared from rabbit-muscle:

Determination of the concentration of succinic acid causing decolorisation in the shortest time.

Mg. of potassium succinate in total volume of 3 cc.	Decolorisation time minutes	Mg. of potassium succinate in total volume of 3 cc.	Decolorisation time minutes
0·024	Not within time of control with muscle alone	1·062	6·5
		1·18	7
0·047	10	1·77	8
0·071	8·5	2·36	10·5
0·094	8	2·95	11·5
0·118	8	3·54	12·5
0·236	7·5	4·72	12·5
0·354	7·5	5·9	13
0·472	7	7·08	14
0·590	7	8·26	16
0·708	6·5	9·44	17·5
0·826	6·5	10·62	20
0·944	6·5	11·8	22

Determination of the concentration of α-ketoglutaric acid causing decolorisation in the shortest time.

Mg. of potassium ketoglutarate in total volume of 3 cc.	Decolorisation time minutes	Mg. of potassium ketoglutarate in total volume of 3 cc.	Decolorisation time minutes
0·292, 0·584, 0·876	Not within time of a control with muscle alone	2·92	42
		3·65	40
		4·38	43
1·168	137	7·30	34
1·46	120	14·6	12
2·19	78		

Higher concentrations could not be tried owing to the small amount of ketoglutaric acid available.

When the optimum amount of succinic acid was now combined with the highest concentration of α-ketoglutaric acid, the time required for complete decolorisation was no shorter, in fact, slightly longer than the shortest time for succinic acid acting alone.

Combination of succinic and α-ketoglutaric acids.

	Mg. in total volume of 3 cc.	Decolorisation time minutes
Potassium succinate	0·826	7
„ ketoglutarate	13·59	

A similar experiment was carried out with an extract made from calf-muscle, the results of which are summarised below.

Concentration of donator. Mg. in total volume of 3 cc.			
Potassium succinate	Potassium α-hydroxyglutarate	Potassium α-ketoglutarate	Decolorisation time minutes
0·029	—	—	Not within time of a control with muscle alone
0·047	—	—	74
0·118	—	—	7·5
—	13·3	—	61
—	—	13·1	20
0·029	13·3	—	19
0·047	13·3	—	11
0·118	13·3	—	7·5
0·029	—	13·1	23
0·047	—	13·1	22
0·118	—	13·1	34

From such experiments it would appear that:

(1) As the concentration of donator substance is increased, the time for complete decolorisation of a fixed quantity of methylene blue diminishes extremely rapidly, and further increase of donator beyond this optimum value causes a lengthening of the decolorisation time.

(2) The optimum time for α-ketoglutaric and α-hydroxyglutaric acids appears to be in very high concentration.

(3) Very slight changes in the concentration of donator may produce very great differences in the decolorisation time; when the concentration is very low there seems to be even inhibition of the process of decolorisation by the muscle itself. This is seen in the cases of succinic acid in a concentration of 0·024 mg. and α-ketoglutaric in concentrations of 0·292, 0·584 and 0·876 mg.

(4) It is hardly to be expected that a comparatively slow reaction such as that of α-hydroxyglutaric or α-ketoglutaric acid would show much detectable effect on a rapid reaction like that of succinic acid.

(5) It would appear from the results of such experiments that the optimum time for reduction in the presence of succinic acid cannot be diminished, while, provided that the combined reaction be an active one, the optimum time for decolorisation is prolonged, just as in the case where the concentration of succinic acid alone is increased.

The finding that low concentrations of substances acting as donators would inhibit the spontaneous decolorisation by the muscle suggested that malic acid, which hitherto had always been found to prevent decolorisation, might, in suitable concentration, act as an activator for the process. On investigation of this point it was found that, for extracts made from rabbit-muscle and ox-muscle: (*a*) the inhibiting action of malic acid on the reduction of the methylene blue was exhibited in concentrations as low as 0·2 cc. of *M*/100 solution of the acid in a total volume of 3 cc.; (*b*) at certain high concentrations [about 0·4 cc. of *M* solution in a total volume of 3 cc.] there was undoubted but incomplete decolorisation; (*c*) in accordance with observation (*b*), when various concentrations of malic acid were combined with the optimum concentration of succinic acid, complete decolorisation could be obtained at two stages in the series, first, if the concentration of malic acid were very small, and, secondly, some decolorisation could be obtained with very high concentrations of the malic acid.

The results suggested further that some of the substances tried in only one concentration and hitherto classed as not donators, might act as such if the concentrations were suitable. Glutaric, glutamic, *l*-tartaric, acetic, and fumaric acids were all tried in varying concentration; in no case was the decolorisation time shorter than that of a control with muscle alone, but in concentrations higher than 0·1 cc. of *M* solution in a total volume of 3 cc., inhibition of the spontaneous reduction by muscle alone was a general effect.

The strong inhibiting action of fumaric acid on the succinic acid reaction suggested that its combination with other donators might yield some information on the specificity of the enzymes. Fumaric acid was found to inhibit, to a certain extent, the decolorisation in the presence of both α-hydroxyglutaric and α-ketoglutaric acids.

These methods of investigation were now abandoned for two reasons. As previously stated, it is not to be expected that the slower reactions would show much effect on the more rapid ones, and, secondly, an investigation of the times for complete decolorisation of the *same* quantity of methylene blue by *different* concentrations of donator does not give a true indication of the course of the reaction. This will be apparent from a closer study of figures taken from two experiments where succinic acid was used as donator substance.

Experiment A. Optimum time—6·5 minutes—obtained when 0·708 mg. of succinic acid was present. The hydrogen removed from the succinic acid and transferred to the methylene blue [as calculated from the amount of methylene blue decolorised] was 2·6 % of the total hydrogen removable from all the succinic acid present, provided it all changed to fumaric acid. This represents a reduction at the rate of 0·4 % per minute.

In the same experiment a time *longer* than the optimum—8·5 minutes—was obtained when 0·071 mg. succinic acid was present. The hydrogen removed in this case equals 25 % of the total removable or a reduction at the rate of 3 % *per minute.*

Experiment B. Optimum time—13 minutes—obtained when 0·708 mg. succinic acid present, a reduction of 1·6 % of the total, or reduction at the rate of 0·13 % per minute.

In the same experiment 102 minutes were required when 0·024 mg. succinic acid were present. This represents a reduction of 51 % of the total or at the rate of 0·5 % *per minute.*

It was therefore thought that a better method would be to vary the quantity of methylene blue present, while keeping the concentration of donator substance constant. The times for complete decolorisation could then be compared with the amounts of methylene blue decolorised, which, presumably, represents the transportation of a certain amount of hydrogen. Such a comparison would then give a truer picture of the course of the reaction. The method, it must be remembered, will not give absolutely accurate results, because, the degree of evacuation of the tubes may not be originally quite the same in each case, and, while tubes that had obviously leaked were discarded, a small undetectable leakage may occur which will materially alter the result, especially where the experiment is of long duration; further it is impossible to determine with absolute accuracy when the contents of a tube are completely decolorised. The effect of these errors is, of course, diminished by the large number of observations made.

Extracts prepared from rabbit liver were employed for the following experiments, because such extracts react with a larger number of donators. In the case of liver extracts, it is more difficult to reduce by washing the activity of the decolorisation by the tissue alone, but a sufficient differentiation can be obtained between the spontaneous decolorisation and the decolorisation in the presence of a donator.

Series of tubes were prepared with the same amount of enzyme and the same concentration of donator in each, but with gradually increasing quantities of methylene blue. The times for complete decolorisation were then compared with the quantity of methylene blue decolorised, and hence the amount of hydrogen transported, in each case. The following table shows the series of quantities employed in conducting such an experiment with lactic acid as donator.

Decolorisation in absence of any donator.

Extract 1 cc.						
Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125
Time for decolorisation (minutes)	12	21	48	106	217	Not decol. in 5 hours

Decolorisation with succinic acid as donator.

Extract 1 cc.; succinic acid as K salt, 0·4 cc. $M/10$ solution in total vol. of 3 cc.							
Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125	6·250
Time for decolorisation (minutes)	4	7·5	13·5	23·5	37	51	77

Decolorisation with lactic acid as donator.

Extract 1 cc.; lactic acid as K salt, 0·4 cc. *M*/10 solution in total vol. of 3 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125
Time for decolorisation (minutes)	6·5	12	24	67	118	Not decol. in 5 hours

Decolorisation with both succinic and lactic acids as donators.

Extract 1 cc.; succinic acid as K salt, 0·4 cc. *M*/10 solution in total vol. of 3 cc.; lactic acid as K salt 0·4 cc. *M*/10 solution in total volume of 3 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125	6·250
Time for decolorisation (minutes)	5	8	15	24·5	37·5	49	82

Decolorisation with double quantity of succinic acid.

Extract 1 cc.; succinic acid as K salt, 0·8 cc. *M*/10 solution in total vol. of 3 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125	6·250
Time for decolorisation (minutes)	8	13	22	31·5	43·5	67	105

These results are presented graphically in graph I. A similar series of observations was made for each of the following: citric acid (graph II); α-keto-glutaric acid (graph III); formic acid (graph IV); and tartronic acid (graph V).

Graph VI shows the rapidity of decolorisation of methylene blue with four different extracts in the absence of any added donator. Owing to the comparative slowness of this spontaneous reaction, this graph is drawn to a different scale from that of the preceding ones.

From a survey of these graphs it will be noted that:

(1) The graph of the rate of decolorisation by muscle alone, *i.e.* in the absence of any added donator, is approximately a straight line.

(2) In the presence of a donator substance the graph of the decolorisation-rate does not resemble that of an ordinary enzymic reaction.

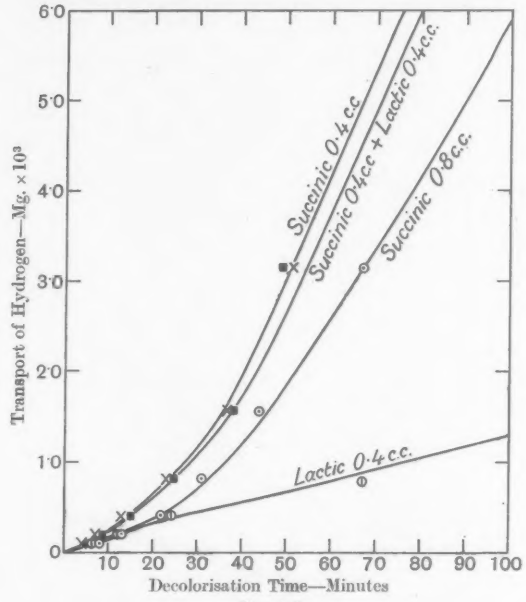
(3) The greater the quantity of methylene blue present the more rapid the decolorisation. This effect is shown to an extreme degree in the case of citric acid where the tubes with least methylene blue took somewhat longer to decolorise than those with slightly greater amounts.

Decolorisation with citric acid as donator.

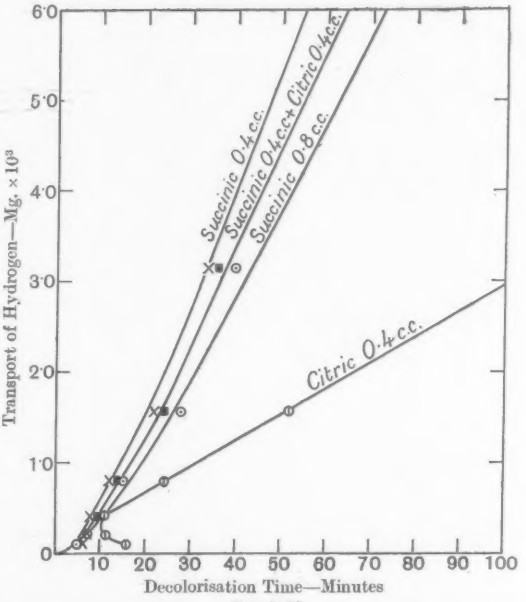
Extract 1 cc.; citric acid as K salt, 0·4 cc. *M*/10 solution in total vol. of 3 cc.

Hydrogen removed as calculated from meth. blue decolorised. Mg. $\times 10^3$	0·098	0·196	0·391	0·782	1·563	3·125
Time for decolorisation (minutes)	16	11·5	11	24	52	105

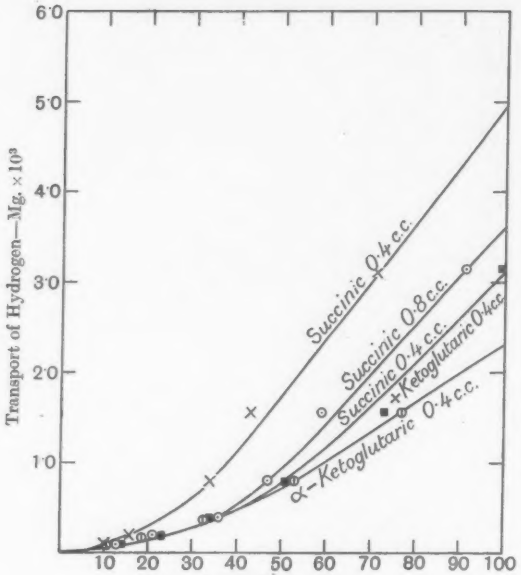
In this case the liver was rapidly washed in order to preserve the effect of citric acid as a donator, and hence the spontaneous decolorisation by the muscle may have been taking a comparatively large part in the reaction.



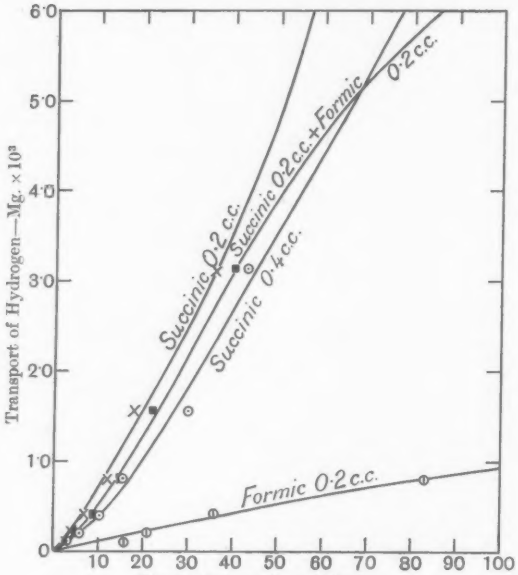
Graph I



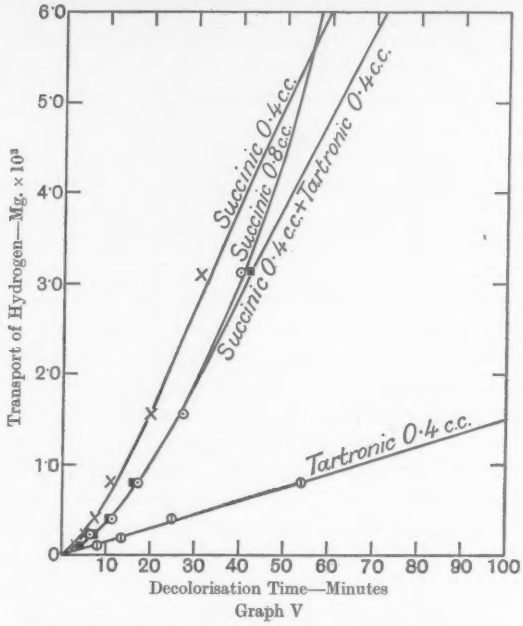
Graph II



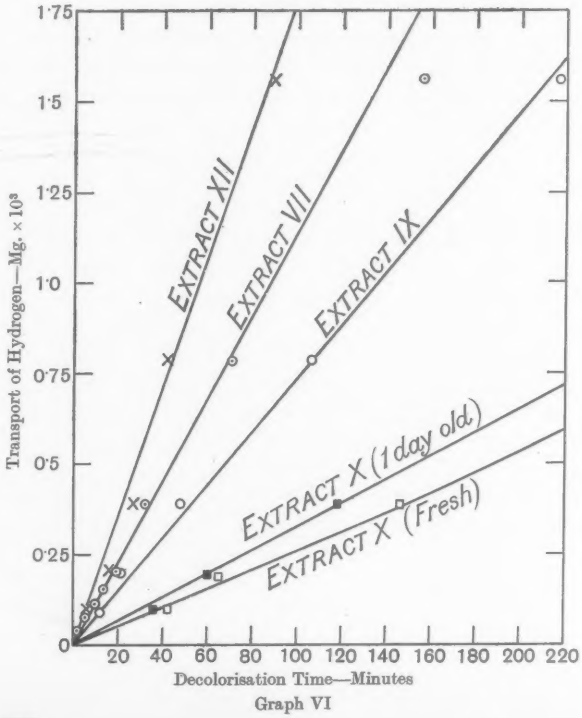
Graph III



Graph IV



Graph V



Graph VI

(4) All the graphs are similar in this respect, that combination of another donator with succinic acid gives a curve tending towards the same form as that given when the concentration of succinic acid is increased.

Conclusions.

1. The hypothetical enzyme acting on the various donators is the same for all, though acting in different degrees with different donators, and being able to discriminate between very closely related substances, *e.g.* α- and β-ketoglutaric acids.

2. The reduction of methylene blue by the muscle alone is a process differing from the reduction which occurs in the presence of a donator substance like succinic acid; the graphs are of different form and it has several times been observed that, as the extract ages, the spontaneous reduction may increase in rapidity while there is little change or a diminution in the rapidity of decolorisation under the influence of a donator.

3. The form of the graphs obtained, both for donator reduction and spontaneous reduction, do not resemble the graphs of ordinary enzyme reactions, and hence it might be suggested that methylene blue does something more than merely play the passive rôle of hydrogen acceptor. In view of the fact that the greater the amount of methylene blue present in the test, the higher the proportional speed of the reaction, it is perhaps possible that the methylene blue actually takes some part in the various changes.

In conclusion, I should like to express my best thanks to Prof. Thunberg for his helpful advice and criticism during the course of the work, and also for the many kindnesses shown to me by him and his staff during my residence in Lund.

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XV. NOTE ON THE SULPHURIC ACID TEST FOR LIVER OILS.

By ARTHUR HARDEN and ROBERT ROBISON.

From the Biochemical Department, Lister Institute.

(*Received January 15th, 1923.*)

The well-known test for liver oils, which consists in the production of a blue or purple coloration on agitating a solution of the oil in carbon disulphide or chloroform with a drop of concentrated sulphuric acid has generally been attributed to the presence in such oils of cholesterol and lipochromes. Recently, however, Drummond and Watson [1922] have drawn attention to the parallelism occurring in a long series of oils between the production of this coloration and the presence of vitamin A, and they conclude that the substance, to which the formation of the coloured compound is due, is neither cholesterol nor, probably, a member of the lipochrome pigments. The general resemblance between this test and that of Pettenkofer for bile acids or Mylius's modification, in which the sugar is replaced by a solution of furfuraldehyde, suggested to us that the reaction might be of the same type as in these latter, namely, a condensation between two compounds under the influence of sulphuric acid. Cholesterol might thus be involved in the reaction though not of itself capable of giving the test, while the second substance, which must also be present in the oil, might be a derivative of furfuraldehyde or other compound of similarly reactive nature. Such a reaction occurs in Neuberg and Rauchwerger's [1904] test for cholesterol in which δ-methylfurfuraldehyde, formed from rhamnose, is employed, a red coloration being produced under the influence of concentrated sulphuric acid.

The purple coloration given by liver oils can, we find, be closely simulated by adding a drop of concentrated sulphuric acid to a light petroleum solution containing both cholesterol and furfuraldehyde or ω-hydroxymethylfurfuraldehyde. Chloroform may also be used as a solvent but unless the solutions are carefully dried, several drops of sulphuric acid will be required to produce the coloration. Further, the addition of furfuraldehyde to such oils as butter, which of themselves give only a faint coloration, causes the production of a very intense purple colour on adding the sulphuric acid.

So far all attempts to obtain furfuraldehyde, or some compound which could replace it in this reaction, from coal fish oil by distillation with steam or under reduced pressure and in other ways have been unsuccessful.

The reaction provides a delicate test for cholesterol, a faint purple colour being slowly given by 0·1 mg. in 5 cc. light petroleum containing excess of furfuraldehyde, while with 0·5 mg. cholesterol the coloration is immediate and pronounced.

Since these experiments were carried out a paper by G. S. Whitby [1923] has come to our notice, in which somewhat similar tests for cholesterol using formaldehyde are described and in view of this we do not propose to continue the work from this aspect.

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XVI. ESTIMATION OF SUGAR IN THE BLOOD.

By EDWIN GEORGE BLEAKLEY CALVERT.

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(*Received November 15th, 1922.*)

For some years past much attention has been directed to the determination of carbohydrate tolerance by observing the variations that occur in the sugar-content of the blood after the ingestion of a known amount of sugar. The value of this method is now universally recognised. It is not intended in this paper to treat the subject in a general way, but merely to consider the actual estimation of sugar as required for the above purpose.

The usual procedure is to determine, first of all, the percentage of sugar in the blood at the "fasting level," then to administer a dose of 30–50 g. of glucose and make further estimations, say, every 20 to 30 minutes for two hours.

Micro-methods are practically always employed, the blood being obtained from a prick of the finger. After trying a number of these methods, it was found that those of Bang, MacLean, Folin and Wu, and Mackenzie Wallis and Gallagher were the most satisfactory. Considerable time and labour are required, however, for the routine performance of any one of these.

For some time past the writer has been endeavouring to simplify existing methods, especially from the clinical standpoint, and to increase, if possible, their accuracy. After a number of investigations, it was finally decided to use the rationale of the method of Folin and Wu [1918, 1920], and Mackenzie Wallis and Gallagher [1920] as a basis for this purpose. The process evolved has now been in use for some time and has proved to be most satisfactory. It differs in so many points from the original—in the collection and dilution of blood, the type of standard colour, the size of the boiling-tube, the curve of correction for copper reduction, etc.—that a full description is necessary.

Method of obtaining blood.

An amount of blood sufficient for accurate estimation of the sugar-content can be obtained from a single prick of the finger. The blood is collected in a small platinum capsule, specially designed for the purpose. This has been found to be the simplest and most efficient way of securing the sample. The collection is made with the greatest ease and the sample of blood is greater in amount than that which can be conveniently obtained by the blotting-papers in general use. It is considered preferable to weigh the small quantity of blood on a torsion balance, than to measure it in a pipette.

Pipettes must be kept scrupulously clean, otherwise blood will probably not run into them. The use of the capsule avoids this trouble.

The platinum capsule is held by a small pair of cross-action forceps (both being shown full size in Fig. 1), and is then hung on the hook of the torsion balance and weighed.

It has been found throughout many blood-sugar estimations, that the weight of the capsule has not varied in the slightest degree; numerous weighings can therefore be omitted. A capsule weighing about 170–200 mg. has proved most suitable.

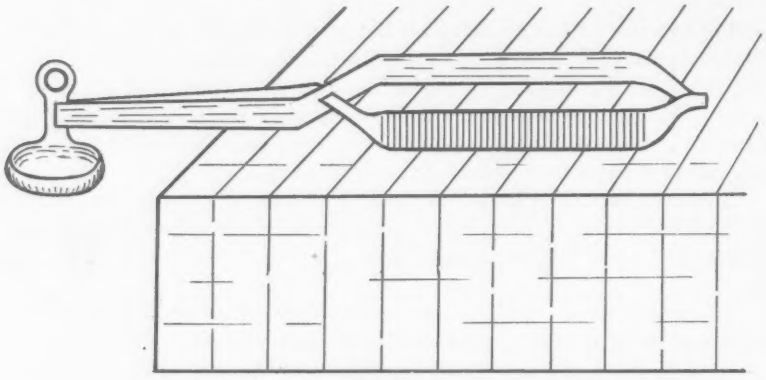


Fig. 1.

The hand of the patient is washed in warm water and dried. Then the thumb (for instance) is cleansed with ether. The patient is instructed to swing the arm, keeping the hand as low as possible and further congestion is caused by winding at once a piece of rubber-tubing round the proximal part of the digit. A prick is made with a bayonet-pointed needle to the side of, and just above, the nail. The thumb is then turned over and the blood made to fall in drops *directly* from the point of puncture into the capsule (held by the forceps) by compression of the pulp and nail. The capsule and blood are weighed immediately. One should obtain 200, or more, mgm. blood; generally it is quite easy to collect up to 270 mgm. It has been found necessary to emphasise: (*a*) that the hand should be warm and (*b*) the thumb thoroughly congested in order to secure the sample in the most convenient way. *Without delay* the capsule and blood are dropped into a short, wide test-tube (see list of apparatus) containing 7·3 cc. of distilled water. A turn or two of the test-tube is sufficient to mix completely the blood and water; none of the blood clings to the capsule.

The lengthy process of sugar extraction, required in blotting-paper methods, is, of course, unnecessary here. In a couple of minutes the mixture becomes clear and then one proceeds immediately to remove the proteins and other interfering substances by using the tungstic-acid-precipitation method of Folin

and Wu. Accordingly 0·3 cc. of 10 % sodium tungstate solution is added and directly afterwards 0·3 cc. of $\frac{2}{3}$ *N* sulphuric acid. This makes the volume up to 8 cc. (0·1 cc. being allowed for blood, less evaporation during expt.). It should be well mixed to ensure full precipitation; a reddish-brown or chocolate-coloured precipitate is quickly formed. The whole is now filtered through starch-free filter paper, the No. 30 Whatman filter paper being the best for the purpose. By pouring in about half of the solution and waiting until the paper is completely moistened before adding the remainder, one obtains the maximum amount of absolutely protein-free filtrate. The filtrate is watery-clear, and from it one determines the percentage of sugar in the blood.

Principle of method of estimating sugar content of blood.

As in the other methods mentioned, the power of glucose to reduce an alkaline solution of copper is utilised. By the addition of phospho-molybdic acid to the cuprous oxide so produced, a clear deep-blue solution is obtained, any unreduced copper being at the same time decolorised. The depth of colour is a measure of the amount of reduction and consequently of the percentage of sugar present. This estimation is made by comparison in a colorimeter with the depth of colour similarly produced from a solution of glucose of known strength. A special feature, however, of the present method of sugar estimation is the simplification of the standard used.

The standard colour used in the colorimetric estimation of sugar.

The present method of producing the standard colour is a fixed procedure requiring frequent repetition. To simplify matters it was decided to replace the standard solution by a glass, or glasses, of exactly the same shade of colour. This was easily accomplished and a perfect matching of colours obtained. The colorimeter reading gave the value of the glass in terms of sugar solution. It is evident that one can afford to devote much more time and care to this single determination than to the routine preparation of standards, which are so liable to vary one from another. Several readings of the colorimeter ensure that an accurate value has been obtained for the particular glass used. In the construction of curves this fixed basis is of special importance. The technique will be described presently.

Apparatus[1] *required:*

(1) A good colorimeter, *preferably the Kober pattern.*

(2) A torsion balance.

(3) Special platinum capsule for collection of blood.

(4) Small pair of cross-action forceps, with smooth gripping surfaces, for holding capsule (see Fig. 1).

(5) Several test-tubes (approximately 2·5 × 8·5 cm.) for dilution of blood and precipitation of its proteins.

[1] The glass-discs are supplied by Messrs Baird and Tatlock (London), Cross Street, Hatton Garden, E.C.; the remainder of the apparatus by Messrs Gallenkamp & Co., 19, Sun Street, E.C. 2

(6) Whatman starch-free filter papers, No. 30 (7 cm. diameter).

(7) Special resistance-glass boiling-tubes, with 7 cc. bulbs and graduated for 12·5 cc. (of same pattern as the Folin and Wu and Mackenzie Wallis tubes; these, however, have only 4 cc. bulbs).

(8) Pipettes of the following capacities: two 1 cc. pipettes graduated in 1/50ths for the 10 % sodium tungstate and $\frac{2}{3}$ *N* H_2SO_4 solutions; two 2 cc. ordinary bulb-pipettes for the copper and phospho-molybdic acid solutions; one 5 cc. pipette for tungstic acid filtrate.

(9) Burette of 25 cc. capacity for distilled water.

(10) Water-bath (fairly large).

(11) A pair of special coloured glass discs, namely "7·5B," referred to below.

(12) In addition to 10 % sodium tungstate and $\frac{2}{3}$ *N* H_2SO_4 solutions the following (very carefully prepared) are required:

(*a*) *Copper solution.* Dissolve 40 g. pure anhydrous sodium carbonate in about 500 cc. distilled water in a litre cylinder. Then add 7·5 g. tartaric acid and when the latter is dissolved and effervescence has ceased, add 4·5 g. crystallised copper sulphate; dissolve without the aid of heat and make up to 1 litre with distilled water. Keep in a dark-coloured bottle. Impurities in the carbonate may give rise to a sediment in the course of a few weeks. If this happens, transfer the clear solution to another bottle.

(*b*) *Phospho-molybdic acid solution.* 35 g. pure molybdic acid are dissolved in 200 cc. of a 10 % solution of sodium hydroxide and 200 cc. of water added. The whole is now boiled for 20–40 minutes or longer until all traces of ammonia have been driven off, as shown by litmus paper held in the vapour. Cool and dilute to about 350 cc. with distilled water and then add 125 cc. of phosphoric acid (85 % strength) and water to 500 cc.; 2 cc. of this solution when added to 2 cc. of the copper solution should produce complete decolorisation.

(*c*) *Standard sugar solution.* When about to determine the sugar value of the coloured glasses, the standard sugar solution is required, made as follows. Dissolve 4 g. of pure powdered glucose in about 500 cc. distilled water and make up to 1 litre (a few drops of toluene will preserve the solution for some time, if necessary); 10 cc. of this made up to 1 litre, is the "standard sugar solution."

Preparation of the blue colour from the standard sugar solution and the matching thereof with the coloured glasses (thus determining the colorimeter-scale values of the latter in terms of the former).

Of the freshly-prepared standard sugar solution 5 cc. (containing 0·2 mgm. glucose) are placed in one of the special boiling tubes and 2 cc. of the copper solution added (the solution now reaching the constricted part of the tube). The tube is shaken so as to mix thoroughly the contents and then placed in a vigorously boiling water-bath for *exactly* six minutes. The volume of water

should be fairly large; no lowering of temperature should be noticed when the tube is introduced. On removal of the latter from the bath, 2 cc. of phospho-molybdic acid solution are immediately added, a clear deep-blue solution resulting. Distilled water is added up to the 12·5 cc. mark and the contents mixed by inverting the tube. This is the standard blue solution, which is to be matched with coloured glass.

For this purpose, a long series of pure blue glass slides[1] (5·1 × 1·8 cm.) was obtained. The 155 slides of the series are accurately graded (·01 to 20·0) as regards depth of colour, and the consecutive colours are so nearly alike that differences can scarcely be detected. It was at once discovered that the colour of the standard solution was not that of pure blue. Investigation proved it to be a mixture of blue and yellow. Accordingly a series of pure yellow glasses, similar to that of the blue, was also employed. After a number of trial experiments, it was found that the blue of the standard solution could be matched exactly by combining one of the blue glasses with a comparatively weak yellow glass. At first it was thought that more accurate estimations of sugar could be obtained by having a number of combinations of blue and yellow glasses, covering the range of colour-intensity derived from blood filtrates, and making use of the approximate combination. Blue glasses of appropriate depths of colour were therefore selected and the necessary correction made with yellow. The latter was determined by placing the standard blue solution (ready for use five minutes after dilution) in one cup of the colorimeter, putting one of the blue glasses underneath the other cup and then adding successively various yellow glasses, until an exact match was effected. At the same time, the cup containing the solution was manipulated so that the colours were also equal in depth. In matching any solution with the fixed depth of colour of the glasses, it is advisable to maintain the cup of the latter at roughly the same level as the cup containing the former. The reading of the cup containing the standard blue solution gives the value of the particular combination of glasses for that solution. After a few trials one gets accustomed to the process and an accurate result is easily obtained. The following are a few of the combinations of glasses with their respective values, as determined by the writer:

No. of glass				Kober colorimeter reading
Blue		Yellow		
15·0	+	1·5	=	29·4
12·5	+	1·2	=	24·3
10·0	+	1·0	=	19·4
7·5	+	0·8	=	15·4

No definite relationship necessarily exists between the grading of the glasses and their colorimeter values.

On employing the above four combinations for each estimation of the sugar-content of numerous blood filtrates and glucose solutions of various strengths, it was found that the same result was obtained with each com-

[1] Lovibond Tintometer Glasses (Tintometer Ltd., Salisbury).

bination of glasses. Therefore only one pair of glasses is required. The 15·0 blue + 1·5 yellow, and 12·5 blue + 1·2 yellow combinations are too deep in colour for low concentrations of sugar and so are unsuitable by themselves. In a few instances even the 10·0 blue + 1·0 yellow may be too dark for the purpose; accordingly as a single combination, the use of the 7·5 blue + 0·8 yellow glasses is advised.

Occasionally when only a very light blue solution is developed from the blood filtrate (e.g. through inability to obtain more than minute quantities of blood, as may possibly occur with infants or markedly debilitated persons, etc.), it will be found helpful to use a pair of discs of lighter colour. "5B" (5·0 blue + 0·5 yellow), having a value of 11·4, can be obtained to meet this contingency.

For ease of manipulation the glasses can now be obtained as pairs of discs; these can be conveniently dropped into one of the cups of the colorimeter. In all cases the values of the glasses should be determined individually in order to allow for the personal equation and for any possible slight variation in the composition of the solutions. Again, it is perhaps advisable to check the value of the discs whenever a fresh stock of copper solution is prepared, as slight variations may possibly occur.

On occasions artificial light may be required for the colorimeter. In this connection a 100 watt Osram daylight lamp[1] (blue) has proved most satisfactory.

Estimation of sugar in blood filtrates by means of the standard glass discs.

One can conveniently pipette off 5 cc. of blood filtrate. This is placed in one of the boiling-tubes and treated in the same manner as the standard sugar solution, *i.e.* 2 cc. copper solution are added and thoroughly mixed; placed for *exactly* six minutes in a boiling water-bath; on removal from bath 2 cc. phospho-molybdic acid solution added immediately; solution made up to 12·5 cc. mark with distilled water and, after the lapse of five minutes, compared in the colorimeter with the glass discs. With very little practice, accurate readings are easily obtained.

Calculation. E.g. A sample of blood collected from a normal person, $3\frac{1}{2}$ hours after a light breakfast.

Weight of platinum capsule	= 171 mgm.
,, ,, + blood	= 395 ,,
Weight of blood	= 224 ,,

The blood was mixed with 7·3 cc. distilled water and 0·3 cc. sodium tungstate solution + 0·3 cc. $\frac{2}{3}$ *N* H_2SO_4 added (= 8 cc., allowing 0·1 cc. for blood, etc. as above). Of the filtrate 5 cc. were used; this represents $\frac{5}{8} \times 224 = 140$ mgm. blood. The blue solution resulting from this filtrate was

[1] Obtainable from G. E. Co., Kingsway, London

found to match the combination of 7·5 blue + 0·8 yellow discs at the colorimeter reading of 25·9. This pair of discs has a value of 15·4, *i.e.* the standard blue solution if set at 15·4 would effect the same match. Now the value of this pair (or of any of the other pairs) of discs represents 0·2 mgm. glucose (the amount contained in the 5 cc. of standard glucose solution employed).

Hence the amount of sugar contained in the 5 cc. of tungstic acid filtrate, *i.e.* in 140 mgm. blood, $= \frac{0{\cdot}2 \times 15{\cdot}4}{25{\cdot}9} = 0{\cdot}1189$ mgm.

This is, however, only an apparent value. A correction depending on the variations of copper reduction, must be made before the true value is obtained. The amended value is 0·139 mgm. (derived from the curve I).

Hence % sugar in the blood $= \frac{0{\cdot}139 \times 100}{140} = 0{\cdot}099$, which is a normal "fasting level."

The correction of apparent values is a matter of importance; it will now be considered in detail.

Curve of correction for copper reduction.

Copper is not reduced proportionately to the amount of sugar present. Therefore cuprous oxide values of solutions of higher or lower sugar concentration than the standard sugar solution do not give accurate values for sugar-content, unless corrected. The writer has worked out a curve of correction for this purpose.

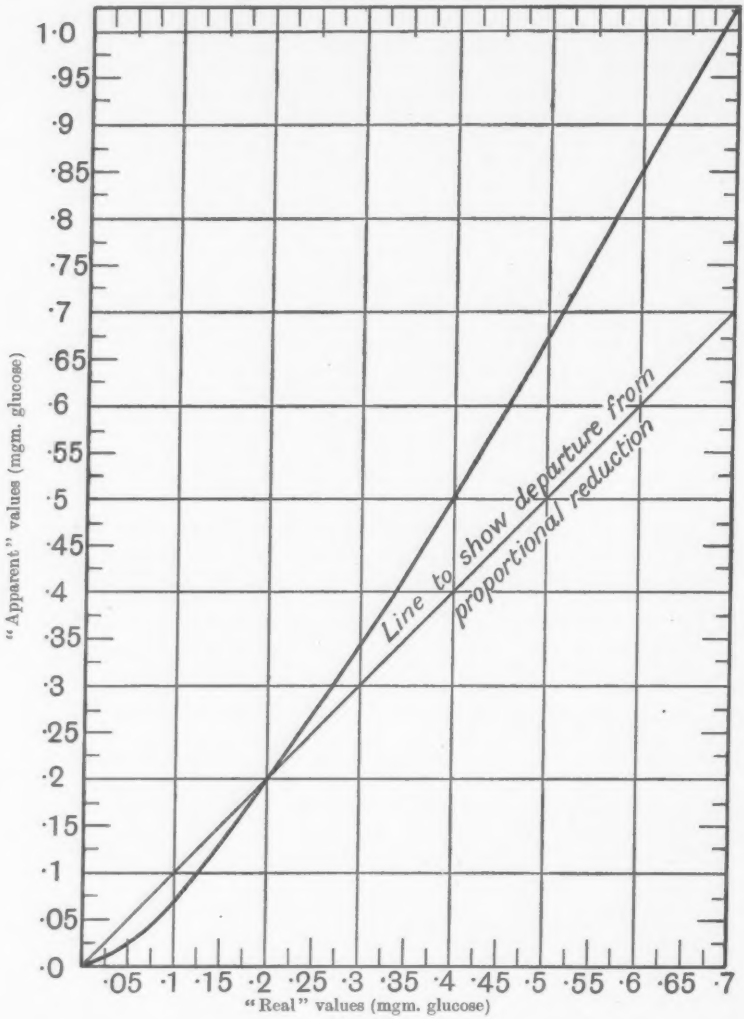
Construction of the curve. As the sugar-content of the blood is estimated from the amount of sugar in 5 cc. of the tungstic acid filtrate, it was decided to employ a series of 5 cc. solutions containing known amounts of pure glucose. One then endeavoured to recover experimentally the sugar content of each of these solutions in exactly the same way as for a blood filtrate. The experi-

Table showing "real" and "apparent" values.

(Mgm. glucose in 5 cc. solution from which the curves are plotted.)

"Real value" (mgm. glucose)	"Apparent value" (mgm. glucose)	"Real value" (mgm. glucose)	"Apparent value" (mgm. glucose)
0·050	0·025	0·525	0·720
0·075	0·045	0·550	0·763
0·100	0·070	0·575	0·808
0·125	0·100	0·600	0·850
0·150	0·133	0·650	0·938
0·175	0·165	0·700	1·025
0·200	0·200	0·800	1·220
0·225	0·233	0·900	1·392
0·250	0·266	1·000	1·567
0·275	0·305	1·100	1·750
0·300	0·343	1·200	1·900
0·325	0·379	1·300	2·008
0·350	0·420	1·400	2·070
0·375	0·460	1·500	2·115
0·400	0·504	1·600	2·140
0·425	0·545	1·700	2·160
0·450	0·587	2·000	2·160
0·475	0·630	2·400	2·160
0·500	0·675	3·000	2·160

mental result is called the "apparent value" in contrast to the "real value"; these have been plotted one against the other. Two curves are given (curves I and II). Curve I is the one intended for use in this method; from the values

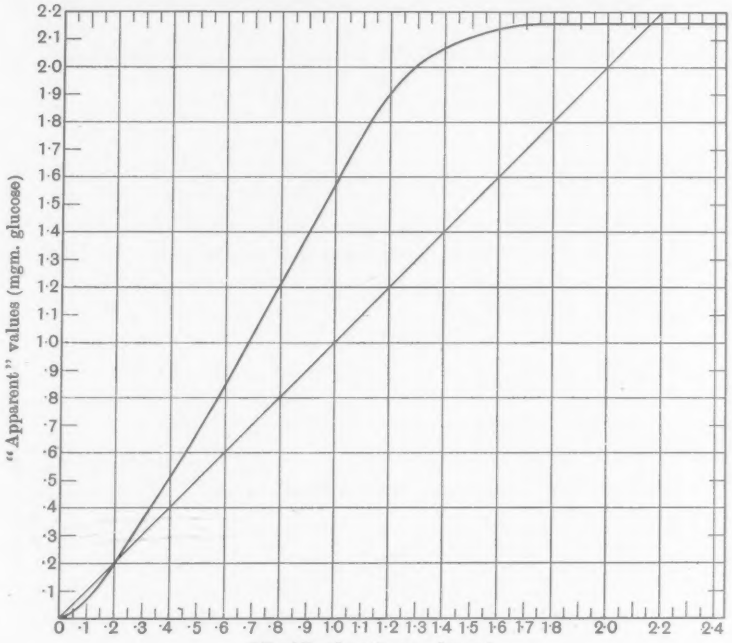


Curve I.

tabulated, it can, with advantage, be drawn to a much larger scale. Curve II is curve I continued by making use of the remaining values; the scale is much smaller.

In preparing the "real value" solutions, great care was taken to avoid error. The experiments were conducted on freshly-prepared solutions; six was found to be a convenient number with which to deal at one time.

First of all, 4 g. of pure powdered glucose was dissolved in about 500 cc. distilled water and the volume made up to 1 litre. If 10 cc. (containing 0·04 g. glucose) of this solution (X) be diluted to 1 litre, then 5 cc. of the resulting solution (Y) will contain 0·2 mgm. glucose. Solution (Y) is really



Curve II.

the "standard sugar solution" already mentioned. By varying the number of cc. of solution (X) various amounts of glucose are obtained in the 5 cc. solutions. Thus, 5, 10, 15, 20, 25, 30, etc., cc. of solution (X) when made up to 1 litre produced solutions, 5 cc. of which contained 0·1, 0·2, 0·3, 0·4, 0·5, 0·6, etc., mgm. glucose respectively. A value like 0·275 mgm. was obtained by preparing solutions containing 0·250 and 0·300 mgm. glucose (*i.e.* 12·5 cc. and 15 cc. solutions (X) used) in 5 cc. and mixing, say, 20 cc. of each; 5 cc. of the mixture contained 0·275 mgm. glucose. The "apparent values" were determined by placing 5 cc. of "real" or known value solutions in boiling-tubes, adding 2 cc. of the copper solution and proceeding as in the case of blood filtrates to make the colorimetric estimations.

An example will make this clear, and, at the same time, demonstrate the equivalence of the several pairs of discs previously referred to, thus illustrating the principle on which the method of using coloured glass standards is founded. In actual practice only the 7·5B pair would be used. (The 7·5 blue + 0·8 yellow combination of discs is conveniently termed 7·5B; similarly one speaks of 10B, 12·5B and 15B.) To determine the "apparent" value of a 5 cc. solution containing 0·30 mgm. glucose, the blue-coloured solution is developed in the usual way and placed in one cup of the colorimeter and compared with a pair of discs placed in the other cup.

The following were the colorimeter readings obtained for the discs:

7·5B = 9·0,	12·5B = 14·1,
10B = 11·3,	15B = 17·1.

Now the values of 7·5B, 10B, 12·5B and 15B are 15·4, 19·4, 24·3 and 29·4 respectively, each value representing 0·2 mgm. glucose. Therefore the "apparent" values given by

$$7\cdot5B = \frac{\cdot2 \times 15\cdot4}{9\cdot0} = 0\cdot342 \text{ mgm. glucose.}$$

$$10B = \frac{\cdot2 \times 19\cdot4}{11\cdot3} = 0\cdot343 \quad ,, \quad ,,$$

$$12\cdot5B = \frac{\cdot2 \times 24\cdot3}{14\cdot1} = 0\cdot344 \quad ,, \quad ,,$$

$$15B = \frac{\cdot2 \times 29\cdot4}{17\cdot1} = 0\cdot343 \quad ,, \quad ,,$$

Mean value = 0·343 mgm. (see table).

As regards the complete curve—curve II—it will be noticed that at three points the "real" and "apparent" values coincide:

at zero,
at 0·2 mgm.,
at 2·16 mgm. ("real" value).

The values are equal at 0·2 mgm. because this is the amount of glucose in 5 cc. of the standard sugar solution, from which the disc-values have been determined. The values are the same at 2·16 mgm. because all the copper in the 2 cc. of stock solution (theoretically amounting to 2·29 mgm. Cu) has been reduced by a 5 cc. solution containing 1·7 mgm. glucose; the "apparent" value at this point is 2·16 mgm., and increase of the glucose used for reduction does not alter it.

Numerous experiments have been carried out which prove the reliability of the curve in enabling one to recover experimentally the percentage of sugar from solutions of known strength. *E.g.* To recover the percentage of glucose in a solution of 0·25 % strength: The platinum capsule was filled with this solution and weighed on the torsion balance, 231 mgm. of the solution being taken.

Capsule + solution were then put into 7·8 cc. of distilled water. 5 cc. of the mixture was placed in a boiling-tube, 2 cc. copper solution added, and the process of estimating the sugar-content continued as already described.

Colorimeter readings obtained:

7·5B (value 15·4) = 7·0. ∴ "apparent" value = $\frac{0{\cdot}2 \times 15{\cdot}4}{7{\cdot}0} = 0{\cdot}440$ mgm.

10B (value 19·4) = 8·8. ∴ "apparent" value = $\frac{0{\cdot}2 \times 19{\cdot}4}{8{\cdot}8} = 0{\cdot}441$ mgm.

∴ Mean "apparent" value = 0·4405 mgm.

If this be left uncorrected the percentage of sugar obtained for the 0·25 % solution would be: $\frac{0{\cdot}4405}{\frac{5}{8}(231)} \times 100 = \frac{44{\cdot}05}{144{\cdot}4} = 0{\cdot}305$ %

(where $\frac{5}{8} \times 231$ or 144·4 = mgm. glucose solution used).

But on making the correction from the curve, the "apparent" value 0·4405 mgm. becomes the "real" value 0·362 mgm., and therefore the amount of sugar found in the 0·25 % solution = $\frac{0{\cdot}362 \times 100}{144{\cdot}4} = 0{\cdot}2507$ %.

Again, the method has been thoroughly tested by recovering added glucose from blood:

For this purpose two capsules were required. Two simultaneous samples of blood were taken, No. 1, 230 mgm.; No. 2, 214 mgm.

On being weighed, No. 1 blood was put immediately into 7·3 cc. distilled water and mixed. No. 2 blood was mixed in 7·1 cc. distilled water. The proteins of No. 1 were precipitated in the usual way and the solution filtered. The capsule now available was employed to weigh out a quantity of 0·25 % glucose solution, 182 mgm. of which were taken.

This was added to No. 2 blood solution. The addition of the precipitants made the volume up to 8 cc.

5 cc. of each filtrate was taken and the sugar content estimated:

(*a*) *Blood alone:*

Amount used = $\frac{5}{8} \times 230$ = 143·75 mgm. blood.

Using 7·5B the reading was 18·3.

∴ "apparent" amount sugar in 143·75 mgm. blood = $\frac{0{\cdot}2 \times 15{\cdot}4}{18{\cdot}3} = 0{\cdot}1683$ mgm.

= 0·1780 mgm. (corrected from curve I).

∴ sugar in the blood = 0·1238 %.

(*b*) *Blood + Glucose:*

Amount blood used = $\frac{5}{8} \times 214 = 133{\cdot}75$ mgm. and this contains (using above %)

$\frac{133{\cdot}75 \times {\cdot}1238}{100} = {\cdot}1656$ mgm. glucose.

The 7·5B reading for the blood + glucose = 5·2.

∴ "apparent" amount glucose in the mixture = $\frac{0{\cdot}2 \times 15{\cdot}4}{5{\cdot}2} = 0{\cdot}592$ mgm.

= 0·452 mgm. (corrected).

Now amount glucose solution used = $\frac{5}{8} \times 182$ = 113·75 mgm.

This amount therefore contains 0·4520 − 0·1656 = 0·2864 mgm. glucose.

∴ amount of glucose in the 0·25 % solution = $\frac{0{\cdot}2864 \times 100}{113{\cdot}75} = {\cdot}2519$ %.

In cases of hyperglycaemia the importance of the curve correction is still more noticeable than with normal blood.

With regard to the solutions used in the method it may be pointed out that the copper solution should be made up freshly every four months; the phospho-molybdic acid, $\frac{2}{3}$ *N* H_2SO_4 and 10 % sodium tungstate solutions seem to keep indefinitely. Attention is directed to the quality of the sodium tungstate used. Carbonates are usually present in commerical tungstates. According to Folin and Wu, these should not exceed a certain amount. On titrating 10 cc. of the 10 % sodium tungstate with *N*/10 HCl, using one drop of phenolphthalein as indicator, not more than 0·4 cc. of the acid should be required. The amount of $\frac{2}{3}$ *N* H_2SO_4 used in the precipitation process has been so regulated as to conform to this standard. No difficulty, however, will be experienced if care be taken to obtain tungstate of good quality. The very slight variations that may occur in the tint of the blue colour developed from blood filtrates are sometimes attributable to impurities in the tungstate; nevertheless an accurate colorimeter reading is easily made.

Again, it should be noted that when the blood is properly coagulated by the tungstic acid, the coagulum changes gradually from pink to chocolate-brown. If this change does not occur within a few minutes, the coagulation is incomplete. In such an emergency an extra drop of $\frac{2}{3}$ *N* H_2SO_4 will usually produce the desired effect.

As regards the care of the platinum capsule: it is shaken up in a couple of changes of water in the test-tube in which it remained on filtering the blood precipitate; then it is dipped successively in absolute alcohol and ether; after one passage through a Bunsen flame it is ready for use again.

Summary.

A rapid and reliable method of estimating sugar in the blood has been described. The number of manipulations has been reduced to a minimum, thus making it particularly valuable in routine clinical work.

The following are its main features:

(1) Collection and weighing of blood in a special platinum capsule—simple and accurate:

(*a*) The capsule remains constant in weight; numerous weighings can thus be avoided.

(*b*) In the process of dilution all the blood is immediately diffused, therefore "sugar-extraction" is unnecessary.

(2) Accuracy is gained by making the fullest use of the blood available.

(3) A fixed colour standard in glass. The selection of the standard and the determination of its value are described. The use of the standard (issued as a pair of glass discs—"7·5B") saves much time and labour. Further, it pro-

vides an unchanging basis for the estimations; this is of importance in the construction of blood-sugar curves.

(4) Use of a curve of correction for copper reduction; an appreciable, if not considerable, error in sugar estimation is thereby eliminated.

(5) The technique is so simple that one can carry out concurrently the estimations necessary for several blood-sugar curves.

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XVII. THE SYNTHESIS OF IMINAZOLYLGLYCINE, THE LOWER HOMOLOGUE OF HISTIDINE.

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(*Received January 16th, 1923.*)

ENGELAND [1908] isolated from human urine a small quantity of a picrolonate, melting at 244°, to which he assigned the formula $C_5H_7O_2N_3$, $C_{10}H_8O_5N_4$. From this, by decomposition with hydrochloric acid, he obtained a solution, which gave a red coloration with sodium diazobenzenesulphonate. He concluded from these observations that the substance was the lower homologue of histidine.

It was believed that the synthesis of this amino-acid would be useful, partly as it might to some extent support Engeland's conclusion as to the identity of his substance, and partly because a knowledge of its properties might facilitate its isolation from other natural sources.

The only method available for the synthesis appeared to be that of Strecker in which, by the joint action of potassium cyanide and ammonium chloride, an aldehyde is converted into an amino-nitrile, from which the amino-acid is obtained by hydrolysis. The iminazolyl-4 (or 5)-formaldehyde [Pyman, 1916] was prepared by the oxidation of iminazolyl-4 (or 5)-methyl alcohol, and the latter substance was obtained from citric acid by the method used by Pyman [1911] in his fundamental syntheses of histamine and histidine. The additional experimental details supplied by Koessler and Hanke [1918] were useful in securing good yields in the various stages of this synthesis.

The iminazolylglycine was obtained impure in rather small yield and had to be purified through the picrolonate. The highest melting point of this synthetic salt, 243° (uncorr.) is in close agreement with Engeland's value of 244°. So far as this slender evidence goes, it tells in favour of Engeland's view as to the nature of the substance which he isolated from urine. As was to be expected, iminazolylglycine closely resembles histidine; it does not, however, give Knoop's reaction.

EXPERIMENTAL PART.

Preparation of iminazolyl-4 (or 5)-glycine.

Equimolecular proportions of iminazolyl-formaldehyde (1 g.), potassium cyanide (0·65 g.) and ammonium chloride (0·53 g.) were dissolved in the minimum of water (5 cc.) and allowed to stand at room temperature for 48 hours. The solution gradually darkened in colour and after a few hours became almost opaque. Attempts were made to prevent this by filling the

reaction vessel with nitrogen and hydrogen, but were unsuccessful. It is important that the concentration of the reacting substances be kept as high as possible; on one occasion, when about twice the usual volume of water was used, scarcely a trace of amino-acid was obtained.

The amino-nitrile was not isolated, but was straightway hydrolysed by adding to the reaction mixture an equal volume of concentrated hydrochloric acid and heating on the boiling water-bath for three hours. The liquid was then evaporated to dryness and the residue dissolved in water. Chlorides were precipitated by silver nitrate, in the presence of nitric acid. After filtration, silver nitrate was added in sufficient quantity to give a brown precipitate with barium hydroxide, and the silver compound of the amino-acid, mixed with silver oxide, was filtered off, washed, and decomposed by hydrogen sulphide. The filtrate from the silver sulphide was boiled to expel hydrogen sulphide, freed from barium by sulphuric acid, and evaporated. The resulting crude amino-acid was recrystallised from dilute alcohol. After several recrystallisations it formed sheaves of microscopic colourless plates, which darkened at 220° and melted with decomposition at 254°. It gave Pauly's reaction for glyoxalines, the triketohydrindene reaction for amino-acids, but not Knoop's test for histidine, neither in its original form, nor as modified by Hunter [1922]. It was readily soluble in water, slightly in methyl, almost insoluble in ethyl alcohol.

Nitrogen by micro-Kjeldahl.

4·92 mg. (dried at 120°) gave 1·275 mg. N N = 25·9 %.
Calculated for $C_5H_7N_3O_2$ N = 26·4 %.

The yield was variable and somewhat low: from 1 g. of the aldehyde, 0·25 to 0·50 g. of crude amino-acid was obtained. The *phosphotungstate* crystallises from hot water in glistening rhombic plates.

Preparation of the picrolonate.

The *monopicrolonate* was prepared by adding a hot saturated aqueous solution of rather less than the theoretical quantity of picrolonic acid to a concentrated aqueous solution of the amino-acid [Levene and Van Slyke, 1912]. On cooling, the picrolonate separated in deep yellow crystals which, after recrystallisation from water and from ethyl alcohol, melted and decomposed at 243°—a figure in good agreement with that given by Engeland [1908].

Nitrogen by micro-Dumas.

4·14 mg. (dried at 100°) gave 0·854 cc. N_2 at 11° and 762 mm. N = 24·66 %.
Calculated for $C_5H_7N_3O_2 . C_{10}H_8N_4O_5$ N = 24·2 %.

Estimation of picrolonic acid.

Picrolonates are decomposed by nitron, and as in the case of nitrates and picrates, the nitron compound is practically insoluble in water. The reaction can therefore be used for the estimation of picrolonic acid, and gives satisfactory results even on the micro scale.

Thus 21·09 mg. pure picrolonic acid gave 45·92 mg. nitron picrolonate.

% picrolonic acid = 99·80.

12·16 mg. 4- (or 5)-iminazolylglycine picrolonate gave 17·43 mg. nitron picrolonate.

% picrolonic acid = 65·7.

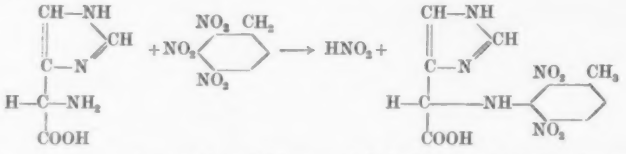
Calculated for $C_5H_7N_3O_2 . C_{10}H_8N_4O_5$ 65·2 %.

Decomposition of the picrolonate.

The picrolonate was dissolved in dilute hydrochloric acid and the free picrolonic acid thus formed was removed by extraction with benzene and finally with ether. The residual solution of the amino-acid hydrochloride was evaporated on the water-bath and the residue was recrystallised from dilute alcohol. It was, however, hygroscopic, and decomposed when attempts were made to dry it at 100°. Similar results were obtained when the hydrochloride was prepared by dissolving the amino-acid in hydrochloric acid.

C.4- (or 5)-iminazolyl-N.2.4-dinitrotolyl-3-glycine.

β- and γ-trinitrotoluene condense with compounds containing a primary amine group, with elimination of nitrous acid [Barger and Tutin, 1918]. In this case, 2 . 3 . 4-trinitrotoluene was used, the 3-nitro-group disappearing in the process.



20 mg. of the amino-acid and 60 mg. of 2.3.4-trinitrotoluene (a considerable excess) were dissolved in dilute alcohol and heated under a reflux condenser for three hours. The liquid gradually darkened to a deep yellow. It was evaporated to dryness, and the unchanged trinitrotoluene removed by repeated extraction with hot benzene. The deep yellow crystalline condensation product remained behind. It was very soluble in water, but insoluble in ethyl alcohol. Recrystallised from dilute alcohol, it decomposed violently at 270°.

The nitrogen content was estimated by a modified micro-Kjeldahl. Eckert [1913] was able to use the Kjeldahl method in presence of aromatic nitro-groups by adding sulphur as a reducing agent. 0·2–0·5 g. of substance was mixed with 0·4 g. of sulphur and heated on the water-bath for one hour with 20 cc. of 30–40 % fuming sulphuric acid. The further procedure was as usual.

In adapting this method for use on the micro-scale, it was found advisable to increase the amount of sulphur to two or three times the weight of substance, and also to heat on the water-bath for two hours. Shorter heating did not give satisfactory results with the substances tried, which, however, as they contained two nitro-groups, afforded a fairly severe test of the method.

6·95 mg. of *N*.2.4-dinitrotolyl-3-histidine were found to contain 1·407 mg. nitrogen. N = 20·3 %.

Calculated for $C_{13}H_{13}N_5O_6$ N = 20·9 %.

3·58 mg. of *C*.4- (or 5)-iminazolyl-*N*.2.4-dinitrotolyl-3-glycine contained 0·7752 mg. nitrogen. N = 21·65 %.

Calculated for $C_{12}H_{11}N_5O_6$ N = 21·8 %.

I wish to express my sincere thanks to Professor Barger for suggesting this research, and for much valuable assistance.

The expenses of this work were partly defrayed by a grant from the Moray Research Fund of this University, for which my thanks are also due.

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XVIII. THE FORMATION OF VITAMIN A IN PLANT TISSUES. II.

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(Part of a Thesis approved for the degree of D.Sc., University of London.)

(*Received November 18th, 1922.*)

Introduction.

Investigators who have used plant tissues as a source of vitamin A in research on its influence on metabolism and growth of animals have frequently observed that the green leaf is richer in this factor than the seed of the plant; and in those plants which form "hearts" such as the cabbage and lettuce, they have found that the green outer leaves are richer than the more or less etiolated inner ones [McCollum, Simmonds and Pitz, 1917; Steenbock and Gross, 1919; Hume, 1921]. No one made any exact comparisons between these two types of tissue until Coward and Drummond [1921] examined etiolated and green shoots of various plants, using very small doses for the feeding tests. They found that shoots which had been exposed to light were very much richer in vitamin A than shoots of the same species grown in darkness; in fact the etiolated shoots in most cases were entirely inactive in the doses used in the tests. In one test of the unsaponifiable matter (shown by Steenbock, Sell and Bluell [1921] to contain the vitamin A if present) from the respective shoots, the dosage must have corresponded to a very great many fresh shoots, and as no growth was obtained from the fraction from the etiolated shoots, it would appear to be possible that sunlight had not only increased the concentration of the vitamin in the green shoot but had, perhaps, even initiated its formation in the plant. This suggested a possible association of the vitamin formation with the photosynthetic activity (carbon assimilation) of the plant and a further series of experiments was planned to determine other conditions of its formation. In the meantime, however, a paper appeared by Wilson [1922] in which he contended that "photosynthesis is not necessary for the production of vitamin A in plants." Hence it seemed desirable to repeat one or two of the former experiments, using the same plant that Wilson had used in his work.

Method for testing a tissue for vitamin A.

The general method of procedure was that described by Drummond and Coward [1920], with the modification described later by Coward and Drummond [1921]. The tissue to be tested was cut from the plant, transferred to a box and carried at once to the animal house where it was put inside the nesting box of the cage. As it was given to the rat early in the morning before the daily ration of basal diet, it was practically always eaten at once and so was not subjected to further influence of light than the experiment demanded. Where the fresh tissue appeared to be unpalatable or poisonous, large masses of it were saponified and the unsaponifiable matter was extracted with ether in the usual way, care being taken to prevent oxidation by carrying out the whole process as far as possible in an atmosphere of carbon dioxide. At each stage of the extraction the separating funnels were filled with carbon dioxide, and during filtration a current of the gas was passed over the filter paper the whole time. The unsaponifiable matter was given directly to the rats by dropping from a glass rod into their mouths and thus the dosage was easily determined. Where seedlings of a definite age were to be fed, seeds were set in plant pots each day and the series used daily when the required age had been attained by those first set.

I. The influence of sunlight.

Wilson drew the conclusion quoted above from the fact that he obtained just as good growth by including 5 % of dried etiolated wheat shoots in his basal ration as by including 5 % of dried green shoots. Apparently this experiment was performed on rats which had not previously become steady in weight on a diet deficient in vitamin A as no preparatory period is mentioned and the experiment was started when the rats' weights ranged from 40 to 60 g. each. It is becoming a matter of common experience that rats whose mothers have received large supplies of vitamin A during pregnancy and lactation, can grow from this weight to maturity on diets containing only minimal traces of vitamin A. Moreover, Wilson's basal mixture contained 20 % of lard, and Daniels and Loughlin [1920] showed that lard could give growth; and Drummond, Zilva, Golding and Coward [1920] have shown very definitely that lard from pigs whose diet has contained vitamin A may itself contain the vitamin in large enough quantities to restore normal growth to young rats when administered to them as 10 or 15 % of their diet. The fact that two of Wilson's rats died on this basal diet after 8½ and 9 weeks respectively is scarcely sufficient proof of its total deficiency in vitamin A. In a later experiment, three of Wilson's rats became steady in weight on his basal diet at body weights of 130, 120 and 107 g. respectively. These are so high that they indicate a rich reserve of vitamin at the beginning of the preparatory period or the presence of some small amount of vitamin in the basal diet. Also, a resumption of growth (gained in this experiment by adding 8 % of dried etiolated wheat shoots to the diet) is usually obtained by much

smaller doses of vitamin A in rats of this weight than are necessary for rats which have become steady at a lower weight. These considerations all seem to indicate that the amount of vitamin A in etiolated wheat shoots must be very small indeed and without a test of wheat seeds on rats with a similar basal diet, it seems scarcely possible to draw a comparison between the vitamin content of etiolated shoots and that of the seeds themselves. It is much to be regretted that, while making this investigation, Wilson did not proceed to make a comparison between etiolated and green wheat shoots in smaller dosage, for he says that "all physiological activity must be more intense in the sprouts grown in the light than in those grown in the dark."

In view of this criticism it seemed desirable to repeat the light experiment on wheat shoots. Two series of sowings of wheat seeds were made on successive days for a period of six weeks, one series being placed immediately in a large airy dark room and the other in a verandah in sunlight, protected above and on two sides by glass. Twelve days after the first sowing the respective shoots were given to experimental rats whose weights had become stationary on our basal diet, and each day for a period of three weeks or longer, one shoot only was given to each rat. The average weight of a fresh etiolated shoot was 0·14 g., its dry weight being 0·012 g. The average weight of a fresh green shoot was 0·12 g., its dry weight 0·011 g. In case even such a small amount of etiolated shoot should give growth, a test was made of one ungerminated seed per rat per day, though in view of our previous findings on wheat seeds this appeared superfluous. It did indeed prove to be so—no growth being shown by any of the four rats and three of them declining in weight and dying. Six rats were used for testing the etiolated wheat shoots, fair growth being obtained in one case, slight growth in two and none in the other three. In marked contrast to these results are those obtained with the green shoots; in each of three rats normal growth was resumed (Fig. 1).

Confirmation of this result was obtained with extracts of unsaponifiable matter from etiolated and green shoots respectively, though the amounts of such matter were very small and only sufficed for ten days' feeding in a dosage of 5 mg. daily. Three rats on the fraction from the etiolated shoots lost between them, 11 g. in body weight. The three rats on the fraction from the green shoots gained 21 g. Each dosage was equivalent to 15 fresh shoots though it is impossible to say what loss occurred during the process of extraction.

A similar test was carried out on shoots of the yellow maize and also on shoots of the white maize. For the former of these, yellow maize purchased at an ordinary corn dealer's was used and for the latter, Carter's white Horsetooth maize was used. The seeds were again set on successive days and when 12 days old used for feeding, but only a small part of the oldest leaf (0·15 g.) was used each day. Striking differences in the growth-promoting powers between the etiolated and green shoots in each case were again found. The etiolated leaf of the white maize was entirely inactive in the quantity fed,

while the same amount of the green shoot produced normal growth; the etiolated shoot of the yellow maize produced growth in one rat, but none in two, while the green shoot produced good growth in three different rats (Fig. 2). Those familiar with the biological test for vitamin A will recognize the irregular behaviour sometimes shown by an individual rat, and hence the necessity of using groups of animals for any one test.

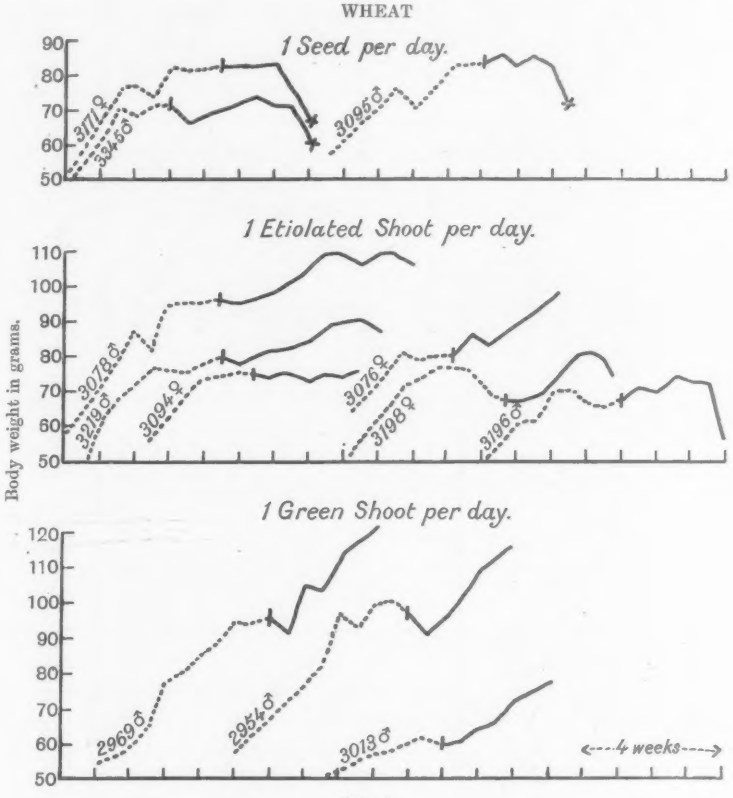

Fig. 1.
Preparatory period on A-deficient diet shown in dotted lines.

Thus, while it would be impossible to deny the existence of very small quantities of vitamin A in etiolated seedlings, it would appear to be established that the amount is increased considerably by exposing the seedlings to sunlight.

It was also thought desirable to examine the influence of electric light on the formation of vitamin A. For this purpose a series of sowings of seeds of *Helianthus annuus* (sunflower) was made. One test was made on the etiolated

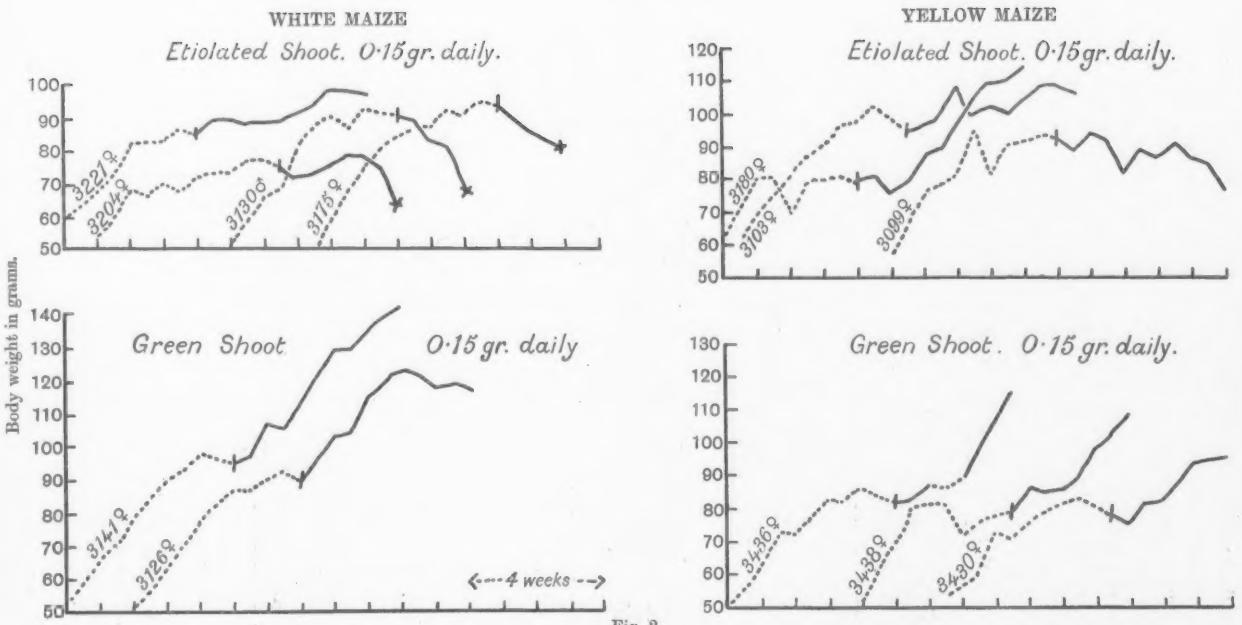

Fig. 2.

Preparatory period on A-deficient diet shown in dotted lines.

seedlings at the age of nine days, a further one on seedlings that had grown in the dark for nine days and then had been subjected to various periods of illumination from an electric light of 32 candle power at a distance of 16 inches. Various trials were made and most marked contrast was obtained with shoots that had been treated for three days with 10 hours' illumination and 14 hours' darkness (when the lights were turned off at night) alternately. The first test with two etiolated seedlings produced only very slight growth, the second test with two illuminated seedlings produced good growth which was only slightly subnormal. A further test on seedlings which had been illuminated as the last set and then kept in the dark for eight days gave, in two rats out of three, growth at least as good as that from the shoots which had been eaten directly after illumination. Hence it would appear that electric light is also effective in increasing the rate of formation of vitamin A, though no comparison of its power relative to sunlight was attempted; and it may also be concluded that the store is not immediately used up by the plant (Table I).

Table I.

	Daily dose	Body-weight in g. 0	7	14	21	28 days	
Helianthus annuus:							
Etiolated seedlings, 9 days old	2 seedlings	58	61	68	75	77	Slight
		60	63	73	75	69	
Green seedlings, after 3 days' electric light	2 seedlings	75	88	96	106		Fairly good
		71	76	80	89		
		63	74	78	80		
Green seedlings, after 3 days' electric light followed by 8 days in the dark	2 seedlings	79	96	105	—	—	Good
		86	96	108	—	—	
Green seedlings, 14 days old, after 1 day in electric light	2 seedlings	91	105	100	95	104	
		90	80	91	96	102	Slight
		70	65	75	81	81	
Tradescantia:	Fresh Mat.						
Grown in Sachs solution	1·0 g.	95	112	115	119	122	
		83	100	116	125	136	Good
		95	95	107	105	117	
Grown in solution without calcium	1·0 g.	72	84	100	105	104	
		67	76	86	90	88	Fairly good
		66	66	68	70	70	

By a further test of growing etiolated seedlings for 14 days in the dark and then exposing them to 10 hours' electric light and 14 hours' dark again, it was shown that no better growth-promoting powers were developed than had been in the nine days old seedlings similarly treated. Thus in vitamin formation in *Helianthus annuus* there is no increased activity through delay in starting it, as Briggs [1920] found was the case with the photosynthetic activity (carbon assimilation) of seedlings of *Phaseolus* (Table I).

II. Independence of carbon dioxide in the air.

As the formation of vitamin A has hitherto been generally associated with those tissues showing at some time photosynthetic activity (carbon assimilation) it seemed advisable to investigate the various factors in this process to see whether they were also factors in the process of formation of vitamin A.

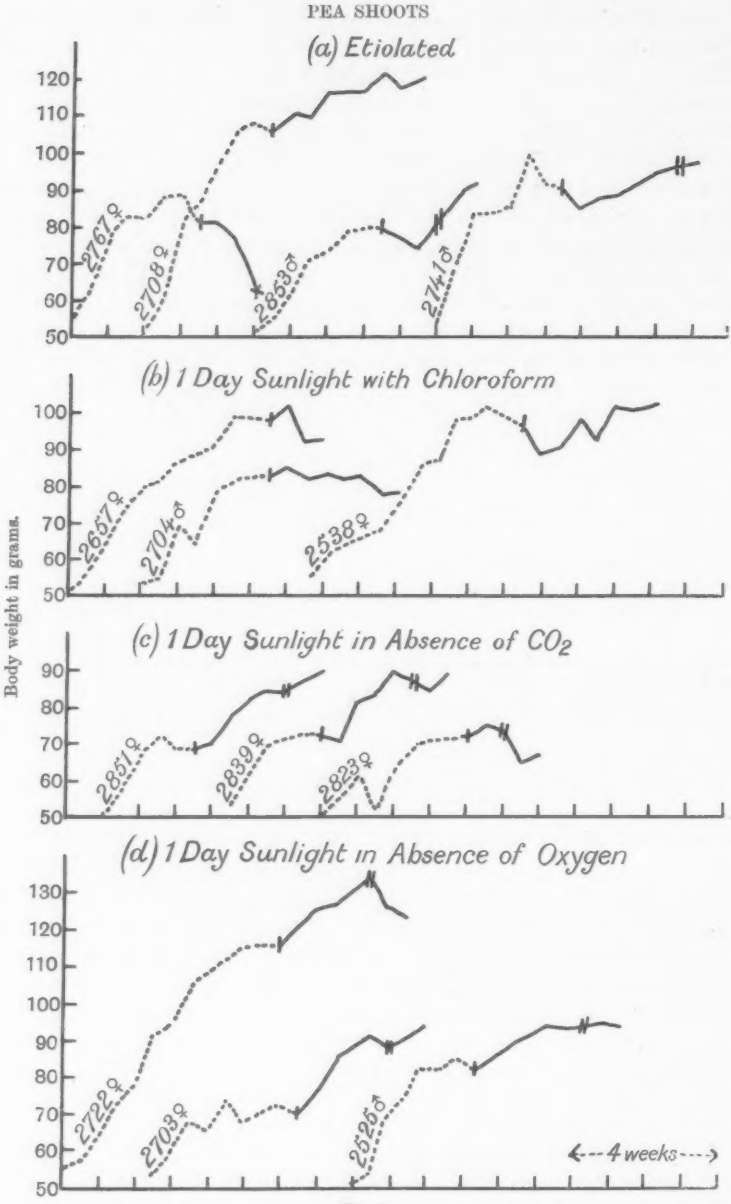

Fig. 3.

Preparatory period on A-deficient diet shown in dotted lines.

The influence of carbon dioxide in the air was first tested. This and the following two tests, together with a control, were run concurrently during May 1922 when, fortunately, there was a succession of sunny days practically without an interruption throughout the experiment. The plan adopted was to cut off shoots of etiolated pea seedlings 12 days old, and place their cut ends in a small tube of fresh tap water in the apparatus each day for 24 hours. The apparatus consisted of a large photographic dish with a layer of mercury, and a bell jar of about 5 litres capacity. Within this stood a wide low dish of 6-inch diameter containing strong potash solution—and the tube containing the cut shoots stood in the middle of this. Thus the shoots would be practically free from carbon dioxide, any that was given out by respiration probably being absorbed by the potash. The whole apparatus stood in a large south window.

A daily supplement of one of these shoots produced growth in rats of the type that is described by Zilva and Miura [1921] as resulting from small doses of vitamin A. That is, the rats grew pretty well for a short time (two weeks), then began to slow down in rate, but on administering two shoots further growth was resumed. One rat only failed to respond to the increased dosage (Fig. 3).

A control of one etiolated pea shoot gave a negative result in three rats. Hence the formation of vitamin A in the plant must be considered as independent of the presence of carbon dioxide in the surrounding atmosphere, if one may ignore the possibility of utilisation of the small quantities admitted to the apparatus in placing the shoot in it, for it might be some time before all the gas became absorbed by the potash (Fig. 3).

III. Independence of oxygen in the air.

To determine whether oxygen is necessary for the formation of vitamin A in plant tissues, an apparatus was set up similar to that used in the last test but a solution of pyrogallol (10 %) in caustic soda (80 %) was used in place of the potash. Most of the air was blown out each day with CO_2 before the fresh seedlings were put into it, in order to ensure as complete an absorption as possible of the remaining oxygen by the pyrogallate. The apparatus stood in the same window as the last one so that it might receive equal illumination.

The results of the feeding test were very similar to the last, three rats resuming growth for about a fortnight, then becoming stationary. One rat grew again on the addition of two shoots instead of one. Thus it appears that vitamin A can be synthesised by the plant practically in the absence of oxygen in the surrounding atmosphere (Fig. 3).

IV. Influence of chloroform in the air.

Very small traces of chloroform in the atmosphere are sufficient to inhibit the process of carbon assimilation in a plant. It was determined to find out whether vitamin formation was affected similarly. Cut ends of etiolated pea

shoots 12 days old were placed in a small jar of water for 24 hours under a bell jar in a south window similar to the one used above, and a small flask of chloroform, open, was also placed under it. The base of the bell jar was not sealed with mercury—fresh air was allowed to enter where it could. The shoots remained apparently healthy and developed a decided green tint.

A ration of one shoot per rat per day so treated failed to restore growth in three rats; hence it may be concluded that chloroform inhibits the formation of vitamin A in plant tissues (Fig. 3).

V. Independence of chlorophyll.

One difference between the shoots deprived of oxygen in Exp. III and those deprived of carbon dioxide in Exp. II was very noticeable each day. The former invariably remained a pure yellow in colour while the latter developed a decided green colour. It is an accepted fact that etiolated shoots will not develop chlorophyll in the absence of oxygen in the air and thus the feeding test of Exp. III leads to another important conclusion. The formation of vitamin A in the plant must be independent of the presence of chlorophyll in the tissues (Fig. 3).

VI. Independence of ultra-violet light.

Another important conclusion may also be drawn from the foregoing experiments. The shoots in two cases, II and III, developed vitamin A when the light which they received had passed through two thicknesses of glass, one the ordinary window glass, the other the thick glass of the bell jar. Hence it may be concluded that the formation of vitamin A is independent of those ultra-violet rays of the spectrum which fail to pass through two thicknesses of ordinary glass—a point in which this process is similar to the more generally examined processes carried on in plant tissues.

VII. Influence of calcium salts.

The close relation between calcium and vitamin A in certain of the latter's activities led the writer to consider whether calcium played any part in the actual formation of the vitamin in the plant, and an attempt was made to cultivate shoots of *Tradescantia* in a medium devoid of calcium. This plant was chosen as it had previously been found very easy to cultivate in water cultures under laboratory conditions. About 200 cuttings were made from normal plants growing in profusion under the staging of a greenhouse. Each shoot, when prepared, consisted of about seven nodes and internodes, the terminal bud and four leaves, the lowest three leaves being cut off and the stem itself cut straight across immediately below the lowest node. The shoots were supported in corks in holes, the stems protected somewhat with a small covering of cotton-wool. Jars of 800 cc. or 1200 cc. capacity were used, four cuttings were put in the former, six in the latter, and the two lowest nodes

of each shoot were submerged in the solution. Half the cuttings were set in normal Sachs solution, half in a solution consisting of 1 g. KNO_3, 0·5 g. NaCl, 0·5 g. NaH_2PO_4, 0·5 g. $MgSO_4$, and two or three drops of $FeCl_3$ sol. to a litre of distilled water. This last was obtained from an ordinary copper still, and was not distilled again. The jars were covered with brown paper, and placed on a window sill with a north aspect.

At first the shoots appeared to grow equally well in the two solutions, practically everyone of them taking root. But a striking difference between the roots was noticed on removing the paper to search for possible moulds. The roots in the Sachs solution had produced masses of root hairs throughout their length, while those in the solution without calcium were devoid of root hairs except for a short distance about half-an-inch long beginning about a quarter-of-an-inch behind each root tip. The root hairs never developed as far as those on the normal roots and apparently died away quite early. This confirms an observation made by Maquenne and Demoussy [1917, 1] in their work on the influence of calcium salts on the growth of seedlings (Plate I, Fig. 2).

When two or three more leaves had expanded on each shoot, the older ones were cut off, and later, when the root systems were well established, the plants were cut down so that only two nodes with undeveloped axillary buds were left. Practically all the plants survived this drastic treatment but the next shoots from the Sachs solution were distinctly more vigorous than those from the calcium-free solution. By this time, three months after the setting of the original cuttings, it was decided to begin the feeding test. 1 g. of the fresh shoot was given to each rat daily and although the shoots grown in the calcium-free solution did not give quite such good growth as those grown in the Sachs solution, yet the growth was very definite in two cases out of three. Maquenne and Demoussy [1917, 2] state that distilled water will dissolve out enough calcium from the glass of the culture vessels to supply the need of seedlings for some time. It would seem probable, however, that after three months with two changes of culture fluid, there would be only traces of calcium left, and it may be concluded that vitamin A can be produced in the presence of only the merest traces of calcium salts in the nutrient solution (Table I).

The question of examining the influence of other elements such as phosphorus, potassium, sulphur, etc., on the formation of the vitamin was also considered, but the difficulty generally experienced in getting cuttings to develop more than one or two more leaves (and no side shoots) in solutions free from these elements, individually, made the matter impossible. Briggs [1922] has just shown that the "photosynthetic activity of plants of *Phaseolus vulgaris* grown in culture solutions devoid of potassium, magnesium, iron or phosphorus is less than that of plants grown in full culture solution." Possibly a lack of calcium also retards photosynthetic activity which would account for the retarded growth of the *Tradescantia* cuttings in the calcium-free solu-

tion. It is impossible to contend that retarded photosynthetic activity in a leaf was the cause of the retarded formation of vitamin A as it has been shown that vitamin A can be formed under conditions ((*a*) lack of chlorophyll, (*b*) lack of CO_2 in atmosphere) in which photosynthesis (carbon assimilation) does not take place to any appreciable extent. And in fact, it is even open to question whether the observed difference in the response of the animals to the two sets of shoots (those grown with calcium and those grown without) was really significant or whether it lay within the limits of experimental error often observed in the only available and decidedly crude test at present used for the vitamin. The main point is that the vitamin can be formed in the presence of only very small traces of calcium in the nutrient solution.

Summary.

1. Light is necessary for the formation of vitamin A in plant tissues.

2. This process can be carried on in the absence of carbon dioxide and of oxygen in the surrounding atmosphere.

3. It is independent of the presence of chlorophyll in the plant.

4. It is also independent of the ultra-violet rays of the spectrum (or such of those as are held back by two plates of window glass) and can be carried on under the influence of electric light in the absence of sunlight.

5. The presence of chloroform in the atmosphere prevents the formation of the vitamin.

6. The almost complete absence of calcium salts from the nutrient solution of a water culture of *Tradescantia* does not prevent the formation of vitamin A in the leaves of the plant.

It is a pleasure to express my gratitude to the Medical Research Council for a grant to defray the cost of this investigation.

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TRADESCANTIA CUTTINGS

Fig. 1
Full Sachs Sol. Calcium lacking
Difference in Vegetative Growth

Fig. 2
Full Sachs Sol. Calcium lacking
Difference in Roots

XIX. THE ASSOCIATION OF VITAMIN A WITH THE LIPOCHROMES OF PLANT TISSUES.

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During the investigation of the conditions of formation of vitamin A in seedlings reported in the preceding paper, the association of vitamin A with lipochromes in plant tissues already emphasised by Steenbock and his co-workers (subsequent references) was being kept in view. Much of the earlier work on vitamin A consisted of the examination of such plant tissues as were commonly in use as food for human beings and farm stock. Steenbock and Gross [1919] found that, of all the roots and underground stems examined by them, the carrot and sweet potato were the only two that contained this vitamin; the other seven they described as a "complete failure." These latter included rutabaga, dasheen, red beet, parsnip, potato, mangold and sugar beet. In each experiment the root formed 15 % of the diet of the test animals, and in the case of the carrot and sweet potato, gave good growth. The minimum dose for growth of each of these was not determined, so that no exact comparison between the two was ever drawn. A rather more quantitative examination was made of the yellow and white maize seeds. When the former constituted 85 % of the ration, the test animals grew and reproduced themselves. The same ration of white maize showed a retardation of the rate of growth in varied lengths of time, which were dependent on the variety of maize used. It was also found that yellow maize in 48 % of the diet gave distinctly poorer growth than the higher ration.

Meanwhile, the close association of vitamin A with the lipochromes in animal fats led many workers to speculate on the possible identity of vitamin A with one of the lipochromes. That there was no foundation for this was, however, shown by Drummond [1919] who prepared pure crystalline carotene from carrots and demonstrated its complete lack of growth-promoting power. Later, also, Drummond and Coward [1920] and Palmer and Kennedy [1921] described many instances of animal fats which were rich in carotene and relatively poor in vitamin A, and others in which the reverse relation held. Steenbock, Sell and Bluell [1921] pointed out that the concentrations of pigment and vitamin A in butter were not closely parallel, though they were

in general agreement, and suggested that this might be due to their having the same source in the green food of the cow. Steenbock, Sell and Boutwell [1921] found that in six different varieties of dried peas, the greater the lipochrome content, the greater was the vitamin A content, though no definite comparison could be made as there is no method of measuring absolute quantities of the vitamin. Steenbock and Sell also found [1922] that the "white sweet potato and the white carrot contained little fat-soluble vitamin, which was in marked contrast to the yellow pigmented varieties. The tops of white carrot roots slightly pigmented with chlorophyll and containing a small amount of yellow pigment were found richer in fat-soluble vitamin than the bottoms containing one-half as much pigment. Green cabbage leaves taken from the heart of cabbage plants which failed to 'head' were found much richer in fat-soluble vitamin than white cabbage leaves in the head. The latter contained only one-tenth as much yellow pigment."

These observations made it seem desirable to examine the lipochrome content of the tissues used in the experiments reported in the previous paper [1923]. Accordingly, the wheat seeds and seedlings of experiment 1 were first tested. 20 g. of the seeds (460 in number) were saponified by heating on a water-bath with 50 % caustic soda for five days and with two volumes of alcohol for two days under a reflux condenser. The alcohol was then distilled off, the mass diluted with two volumes of water and extracted with light petroleum (B.P. 40–70°) three times. The extracts were combined, washed with water six times, always in an atmosphere of carbon dioxide, dried over anhydrous sodium sulphate, evaporated down in a current of carbon dioxide under reduced pressure and made up to 10 cc. exactly. The strength of the solution was estimated by means of a Hellige colorimeter, comparison being made against a 0·2 % solution of potassium dichromate. The concentration of the pigment was determined by reference to curves kindly lent by Dr O. Rosenheim and drawn according to observations made by himself on solutions of the lipochromes of known strength, in confirmation of Willstätter's work on the same point. The curves of carotene and xanthophyll are far from being identical: the curve for xanthophyll is more nearly a straight line than that for carotene. The estimation of the lipochromes in a solution cannot therefore be made exactly without first effecting a separation and then making an estimation of each independently. But where carotene and xanthophyll occur in about the same relative proportions in different tissues, one being largely in excess over the other, a fairly true comparison may be made by using the curve of the lipochrome which occurs in the greater proportion. To determine this proportion, Willstätter's phase test is applied. To a given volume of the light petroleum solution of the mixture, an equal volume of 90 % methyl alcohol is added. The xanthophyll goes into the lower alcohol layer, the carotene remains in the petroleum, and the relative strengths of the solutions may be roughly estimated. The amount of pigment in the seeds was extremely small, 0·02 mg. in the 460 seeds used, and, moreover, the pigment did not give the usual colour

reactions for lipochromes. Wheat seeds were also grown in the dark and light respectively for 12 days and the lipochrome content of the same number of shoots (460) estimated in the same way. In the 460 etiolated shoots were 1·062 mg. of lipochrome (carotene: xanthophyll = 1 : 3) estimated by Willstätter's curves as xanthophyll; while in the 460 green shoots were 1·875 mg. of lipochrome (carotene: xanthophyll = 1 : 3). Some yellow pigment was left in the remaining seeds and roots in both cases (0·064 mg. in the etiolated, and 0·1 mg. in the green), but it did not give the lipochrome reactions with strong sulphuric and nitric acids. The interesting point in the comparison is that the lipochrome content of both lots of shoots was very much greater than that of the seeds from which they had grown, and that the content of the green shoots was, roughly, nearly twice that of the etiolated shoots (Table I).

Etiolated and green shoots of yellow maize contained approximately equal amounts of lipochrome, which was greater than the amount contained in an equal number of seeds (Table I).

Table I.

	Weight in g.	No. of seeds or shoots	Lipochrome tests			Phase test *C* : *X*	Total lipochrome content mg.
			H_2SO_4	HNO_3	I in KI		
Wheat:							
Seeds	20	460	–	–	–		0·02?
Etiolated shoots		460	+	+	+	1 : 3	1·062
Green shoots		460	+	+	+	1 : 3	1·875
Yellow maize:							
Seeds	20	73	+	+	–	1 : 2	0·15
Etiolated shoots		73	+	+	–	1 : 3	0·21
Green shoots		73	+	+	–	1 : 3	0·21
Peas:							
Seeds, dry	26	126	+	+	–	1 : 3	0·11
Etiolated shoots		126	+	+	–	1 : 3	0·02
Shoots without CO_2		126	+	+	–	1 : 3	0·11
Shoots without O_2		126	+	+	–	1 : 2	0·09
Remainder seeds and roots		126	+	+	–	2 : 3	0·065

A similar comparison was made between seeds and shoots of peas treated as in experiments 2 and 3 of the preceding paper; the day on which the shoots were exposed to the light was however not nearly as sunny as any one during the feeding test. The results are summarised and it is interesting to note that there was less lipochrome in the etiolated shoots than in the seeds and the amount of lipochrome remaining in the roots and seeds (not nearly exhausted of the food store by this time) was larger than that of the etiolated shoots. The amounts in the shoots exposed to light (without carbon dioxide and without oxygen respectively) showed a content quite or nearly as great as the dry seeds (Table I). It is at least suggestive to note by the way that the ratio, xanthophyll/carotene, was less in the shoots deprived of oxygen than that in the shoots deprived of carbon dioxide. But the important point to note at present is that lipochromes are present in many of these tissues before the

appearance of the vitamin. A comparison between the absolute amounts of lipochrome present in shoots which will not promote growth and those which will do so once again proves that the activity of the tissue is not measured by its lipochrome content.

Lipochrome content of:		
100 etiolated pea shoots	0·016 mg.	Inactive in dosage of one shoot
„ pea shoots exposed to light in absence of CO_2	0·09	Active „ „ „
„ pea shoots exposed to light in absence of oxygen	0·07	„ „ „ „
„ etiolated wheat shoots	0·23	Inactive „ „ „
„ green „ „	0·4	Active „ „ „
„ etiolated shoots of yellow maize	0·28	Inactive „ „ „
„ green „ „ „	0·28	Active „ „ „

These estimations were made on much larger numbers of shoots than those indicated but have been reduced to 100 to draw the comparison of the lipochromes.

In the meantime, an examination was being made on all available lipochrome-containing tissues from plant growths as varied as possible from a botanical point of view; and a comparison was made according to lipochrome content, growth-promoting power, and calcium content (to verify if possible the finding reported in the preceding paper on the dispensability of calcium salts in the formation of vitamin A).

A polyanthus narcissus was first investigated. The perianth of this variety is a deep rich orange and the corona even darker, but an estimation of the lipochrome content showed that the whole perianth of six lobes contained only slightly more than the corona, a much smaller structure, of the same flower. No calcium was found in 3 g. of perianth, 2 g. corona or in 0·2 g. of the flower sheath; but 0·25 % of the dry weight of the stem was calcium, estimated on 2 g. fresh material. Twenty coronas given to each of two rats daily (put in a small dish in the cage and generally eaten greedily) produced good growth as did also the perianth lobes from 20 flowers to each of two other rats (Fig. 1 and Table III).

A pure "paper-white" narcissus gave slight growth in one of three rats, none in the other two; but an equal dose of the perianth and corona of a "poetaz" narcissus (white perianth and pale yellow corona) gave very definite growth in these same rats. No calcium was found in 3 g. of perianth of the paper-white narcissus though it formed 0·53 % of the dry weight of the stems (estimated on 6 g.) and 1·8 % of the dry weight of the brown sheaths (estimated on 0·4 g.) (Fig. 1 and Table III).

On giving coronas or perianths of a large daffodil (*Narcissus Buonaparte*) to rats, they dropped in weight at once, but on discontinuing this addition, they invariably recovered their previous weight and even grew for a few days. This suggested the presence of vitamin A in this tissue and also some toxic substance. To avoid the effect of the latter, the unsaponifiable matter was extracted from the various tissues and used for the following experiment. By a comparison of the vitamin content of daffodil buds with that of flowers opened in the laboratory (the stems being cut as short as possible) it was

hoped to obtain a confirmation of the previous finding on the formation of the vitamin in a plant tissue in the presence of lipochromes and in sunlight—that is, that there should be no question of its having been transported to the flowers from the leaves. Some growth was obtained on the unsaponifiable matter from 170 buds, but no growth was obtained on the extract from 140 flowers opened in the laboratory, nor on the extract from 140 flowers opened naturally. Possibly the conditions of its storage while waiting for suitable rats for the test accounted for the inactivity but it was too late in the season to repeat the test. Again no calcium was found in the buds (5 g.), in the flowers (5 g.) opened naturally, or in the flowers (6 g.) opened in the laboratory which had been supplied with North London tap-water. But the sheaths (1 g.), leaves (4 g.), and stems (6 g.) contained 0·57, 0·39, 0·39 % calcium, respectively, of their dry weights. This is interesting botanically as no stomata were found in these perianths or in those of any of the other narcissi, yet stomata occurred on the outer (botanically "under") surface of the sheaths and in the stems and leaves. The epidermal cells of the perianths and coronas of all the yellow varieties had very definite conical-shaped outer walls, but they were not so highly developed in the white varieties. Haberlandt [1914] ascribes to this structure the function of concentrating the light on the contents of the cell (Tables II and III).

A chance of testing equally illuminated parts of the same flowers, the one containing much carotene and the other none, occurred in *Narcissus poeticus*—Barr's "White Standard," popularly known as "Pheasant's Eye." Seven hundred of these flowers were obtained, and the white perianths removed without the very small yellow patch that occurs at the base of each lobe.

The coronas were also removed and the two lots saponified separately. The unsaponifiable matter from the white perianth lobes (2·8 g. from 370 g. material) gave no growth in three rats, while the extract from the yellow coronas (0·2 g. from 11 g.) gave very good growth in three rats for eleven days (Fig. 1).

Yellow tulips gave growth on the unsaponifiable matter of their leaves and perianths, the former being stronger than the latter. Again no calcium was found in the perianths though it formed 2·5 % of the dry weight of the leaves. The stems contained no detectable calcium and in this flower there is no sheath. The lipochrome content of the perianths was low, being only 0·005 mg. in 10 g. fresh material (Tables II and III).

Other instances of the association of vitamin A with lipochromes[1] in plant tissue studied in the work are afforded by (*a*) tomato pulp, (*b*) cucumber skin, (*c*) yellow iris flower, the sepals, petals, petaloid stigmas being used in turn, but *not* the stamens or pollen, (*d*) orange juice, (*e*) red capsicum fruit, (*f*) yellow capsicum fruit, (*g*) the calyx of the ripened fruit of the winter cherry (Tables II and IV). In this connection, it should be noted that tomato

[1] A full account of the lipochromes (carotinoids) and their occurrence in animal and plant tissues has just been written by Palmer (1922).

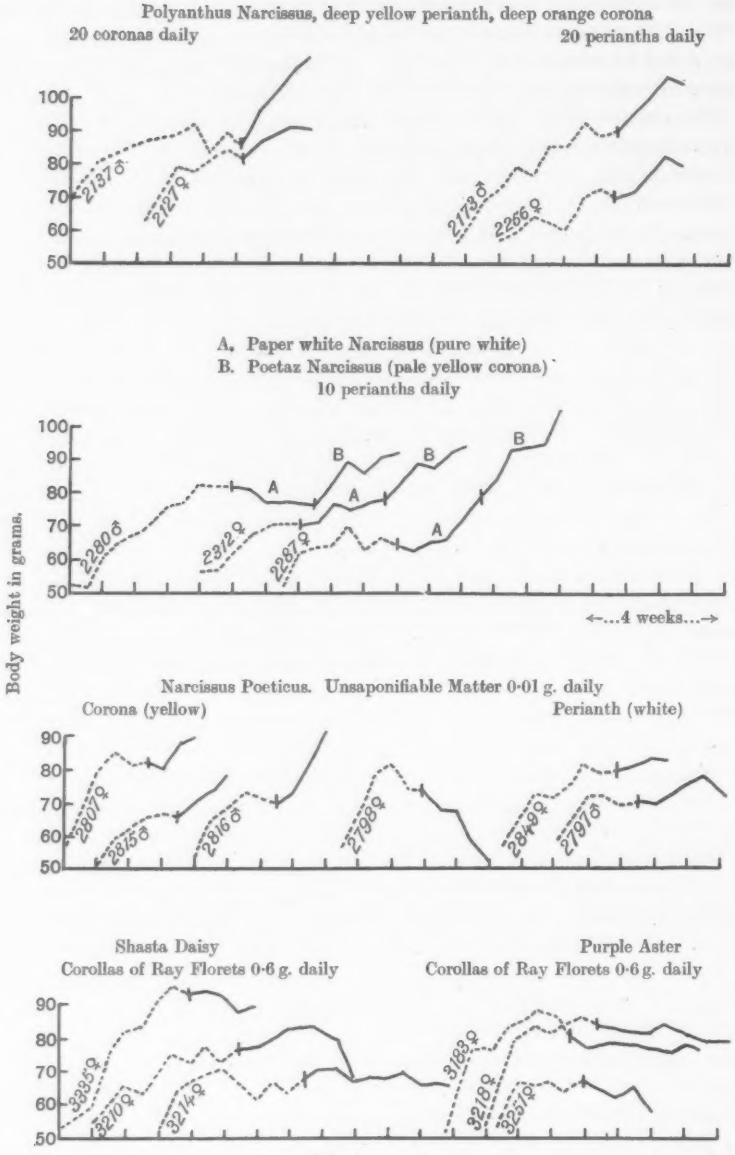

Fig. 1.

Preparatory period on A-deficient diet shown in dotted lines.

fruit has already been shown to contain vitamin A by Osborne and Mendel [1920, 1], that indications of the presence of the vitamin in orange juice have been noted by Osborne and Mendel [1920, 2] and also by Hess, McCann and Pappenheimer [1921].

The absence of vitamin A is associated with absence of lipochrome in (*a*) four varieties of mangolds (the flesh of the root only tested), (*b*) one variety of swede, (*c*) the fleshy part of the cucumber fruit (slight), (*d*) the inflorescence of the cauliflower, (*e*) the corollas (only) of the ray florets of the Shasta daisy, (*f*) the corollas (only) of the ray florets of a purple aster (Tables II and IV, and Fig. 4).

Table II.

	Daily dose. Unsap. matter	Body weight of rat in g. 0	7	14	21	28 days	
Narcissus Buonaparte:							
Perianths of buds	0·01 g.	72	72	82	82	—	Very fair
		82	89	90	94	—	
		98	100	120	124	—	
Perianths of flowers opened naturally	0·01 g.	90	89	—	—	—	Probably none (period too short)
		70	62	—	—	—	
		84	95	—	—	—	
Perianths of flowers opened in lab.	0·01 g.	99	97	97	—	—	None
		76	73	76	—	—	
		72	76	70	—	—	
		81	81	65*	—	—	
Leaves	0·01 g.	74	85	94	—	—	Very good
		102	126	142	—	—	
		89	92	112	—	—	
Yellow Tulip:							
Perianth	0·01 g.	76	74	82	90	—	Slight
		81	89	100	87 (cold)		
		75	74	76	77	—	
Leaves	0·01 g.	81	86	102	105	—	Fairly good
		69	70	79	86	—	
	Fresh matter						
Tomato pulp	0·5 g.	88	97	116	125	130	Fairly good
		79	80	92	99	102	
		82	88	86	95	99	
Cucumber skin	1·0 g.	96	109	116	122	130	Good
		79	91	110	112	112	
		62	60	70	73	70	
Yellow iris, sepals, petals and stigmas	0·2 g.	74	83	—	—	—	Probably good (period too short)
		69	74	—	—	—	
		83	101	—	—	—	
Orange juice	10 cc.	96	103	111	—	—	Good
		85	100	115	—	—	
		83	97	112	—	—	
Red capsicum, fruit	1·0 g.	94	102	116	—	—	Good
		74	82	99	—	—	
Yellow capsicum, fruit	1·0 g.	91	98	116	—	—	Good
		96	111	121	—	—	
Winter Cherry, calyx only	0·1 g.	92	103	115	—	—	Good
		78	84	90	—	—	
		83	96	107	—	—	

Table II *continued*

	Daily dose Fresh matter	Body weight of rat in g. 0	7	14	21	28 days	
Mangolds: Golden Tankard	1·0 g.	98	95	78	—	—	None
		96	109	94	—	—	
		90	88	79	—	—	
Swedes: Red Queen	1·0 g.	75	78	84	—	—	None
		72	75	73	—	—	
		83	84	78	—	—	
Cucumber, fleshy part of fruit	1·0 g.	89	91	90	98	98	Slight
		75	78	84	91	86	
Cauliflower, inflorescence	1·0 g.	96	98	100	—	—	None
		78	81	80	—	—	
	Unsap. matter						
Laminaria	0·01 g.	104	118	113	—	—	Practically none
		105	105	108	—	—	
		105	105	105	—	—	
Fucus vesiculosus	0·01 g.	93	89	102	—	—	Probably none
		76	65	66	—	—	
		77	72	66	—	—	
Lilium candidum, pollen	0·01 g.	80	82	79	—	—	None
		82	92	88	—	—	
		83	88	83	—	—	
Carrot shoots, etiolated	0·8 g.	97	103	106	—	—	Fair
		90	95	101	—	—	
		82	91	91	—	—	
Carrot shoots, etiolated	0·2 g.	89	93	96	—	—	Fair
		85	91	97	102	—	
		97	111	119	119	—	
Turnip shoots, etiolated	0·2 g.	94	94	92	—	—	None
		68	70	66	—	—	
		72	70	63	—	—	
Carrot, roots	0·01 g.	63	63	78	—	—	Fairly good
		56	62	66	—	—	
		61	63	71	—	—	
Turnip, roots	0·01 g.	96	78	65	—	—	None
		86	78	81	—	—	
		71	66	61	—	—	
Polyanthus narcissus, stems	0·01 g.	102	105	—	—	—	Fair
		66	71	72	—	—	
		75	80	86	—	—	

An effort was made in the late summer to find a yellow flower whose pigments were wholly water-soluble, but in five varieties of *Nasturtium,* in *Helianthus annuus,* in the African marigold, in *Ranunculus acris,* in *Lotus corniculatus,* lipochromes were always present. Hence no example of the association of the vitamin with a yellow water-soluble pigment has been traced, and its non-occurrence in the flesh of the mangolds and swedes which contain large quantities of these pigments has been clearly demonstrated, though in this case, of course, light has had no immediate influence.

Another apparent exception to what appears to be a general rule, the association of vitamin A with lipochromes wherever they are exposed to light, was found in *Fucus vesiculosus,* a brown seaweed which grows just below high water mark; patches of this are mingled with patches of *Enteromorpha,* a green

Table III.

	Water	Ash % of dry wt.	Calcium: % of dry wt.	Calcium: estimated on approx. g.	Dose g.	Growth
Polyanthus narcissus:					Fresh matter	
Perianth lobes	90·96	9·5	0	3	1	Good
Coronas	86·61	5·82	0	2	0·5	,,
					Unsap. matter	
Stems	93·90	6·23	0·25	2	0·01	Slight
"Paper-white" narcissus:					Fresh matter	
Perianth including corona	89·49	8·17	0	3	1	Very slight
Stems	90·42	8·3	0·53	6	—	—
Flower sheaths (brown)	12·76	7·7	1·8	0·4	—	—
Narcissus Buonaparte:					Unsap. matter	
Perianths of buds	83·57	3·65	0	5	0·01	Fair
Perianths of flowers opened naturally	89·93	4·55	0	5	0·01	None
Perianths of flowers opened in lab. ...	92·76	6·45	0	6	0·01	,,
Flower sheaths (brown)	49·78	7·35	0·57	1·0	—	—
Leaves	85·87	7·0	0·39	4	0·01	Very good
Stems	92·74	6·05	0·39	6	—	—
Yellow Tulip:						
Perianth	89·45	5·32	0	3	0·01	Fair
Leaves	87·56	9·64	2·52	3	0·01	Good

(It is interesting to note incidentally the occurrence of calcium in those tissues in which photosynthesis (carbon assimilation) has at some time taken place and its apparent absence from those tissues where presumably this process has never been carried on.)

seaweed, on the boulders and rocks of the seashore, but on the groynes built out as breakwaters on the South Coast, the *Fucus* grows distinctly below the green weeds and therefore would be covered by the tide for a somewhat longer period than the green ones. A test on the washed fresh material previously reported [Coward and Drummond, 1921] gave no evidence of vitamin A in *Fucus*, though a green weed *Enteromorpha* had given positive results, and later tests on the extract of unsaponifiable matter of *Fucus vesiculosus* and *Laminaria*, respectively, have also detected no vitamin (Table II). The lipochromes of this species and of *Laminaria* were then examined, the method adopted being as follows.

The fresh material was ground up with solid caustic soda (about 1 g. to 5 g. fresh material) to break down the chlorophyll into components (chlorophyllins etc.) which are insoluble in light petroleum, and also to prevent possible enzyme action. A few drops of ether were also added, and, when necessary, a little silver sand to help to break down the tissue. The mixture was then ground up with anhydrous sodium sulphate to retain water, and light petroleum (B.P. 40–70°) added to dissolve out the lipochromes. In the case of *Laminaria*, the extract was scarcely coloured and a further extract with the same solvent was colourless. Ether was then tried and formed a deep yellow solution which gave a well-defined spectrum 475–450 and 420 to the end. There was only a slight indication of the most characteristic carotene band, *i.e.*

490–480. The solution gave the light blue colour reaction with 30 % hydrochloric acid characteristic of fucoxanthin according to Willstätter—and also the general lipochrome reactions with conc. H_2SO_4 and HNO_3. A second ether extract of the same preparation gave a spectrum with no trace of the first carotene band, but gave the fucoxanthin bands very well defined again. It was concluded that *Laminaria* contained only the merest traces of carotene or xanthophyll, but large quantities of fucoxanthin.

Table IV

	Daily dosage g.	Lipochrome present	Spectrum	Colour reactions with H_2SO_4	Colour reactions with HNO_3	Activity
Tomato pulp	0·5	Lycopin carotene?	Typical Masked by leycopin if present	+	+	Good
Cucumber skin	0·5	Carotene and xanthophyll	Typical	+	+	,,
Yellow Iris, flower	0·2	,,	,,	+	+	,,
Orange juice	10 cc.	Carotene and xanthophyll 4 : 1	,,	+	+	,,
Red capsicum, fruit	0·1	Lycopin carotene? no xanthophyll	,,	+	+	Very good
Yellow capsicum, fruit	1·0	Carotene, very little xanthophyll	,,	+	+	,, ,,
Winter Cherry, calyx	0·1	All carotene	,,	+	+	Good
No attempt was made in this test to find the minimum dosage for growth.						
Mangolds (flesh of root):						
Sutton's Golden Globe	1	–	–	–	–	Poor
Carter's Red Chief	1	–	–	–	–	None
Carter's Imp. Long red	1	–	–	–	–	,,
Toogood's Golden Tankard	1	–	–	–	–	,,
Swede (flesh of root):						
Sutton's Red Queen	1	–	–	–	–	,,
Cucumber, fleshy part of fruit	1	Little carotene and xanthophyll	+	+	+	Slight
Cauliflower, inflorescence	1	–	–	–	–	None
Shasta daisy, corollas of ray florets	0·6	–	–	–	–	,,
Purple aster, corollas of ray florets	0·8	–	–	–	–	,,

(The colour reactions were obtained by drying a little of the light petroleum solution on a glazed tile and adding one drop of the acid. The colour was observed at the edge of the drop of acid before charring took place.)

Fucus, on the other hand, gave a rather stronger solution of lipochromes with light petroleum; carotene or xanthophyll and fucoxanthin (traces only) were identified by means of the spectrum and 30 % HCl reaction, respectively. A further extraction with ether took out far more pigment and gave the carotene spectrum and the fucoxanthin reaction very strongly. This was repeated several times, and always the two lipochromes were obtained. A phase test of the light petroleum solution with MeOH gave a colourless lower layer, so that xanthophyll is not always present, though Willstätter and Stoll [1913] record xanthophyll and no carotene.

Pelvetia behaved like *Laminaria*, containing much fucoxanthin and very little indeed of carotene or xanthophyll.

Thus, although lipochromes are present in two brown seaweeds, fucoxanthin in *Laminaria* which is exposed only at low tide, and fucoxanthin and carotene or xanthophyll in *Fucus*, and both are exposed to a certain amount of sunlight daily, yet neither has apparently produced any vitamin A detectable by feeding tests. The specimens were examined in late summer, when the intensity of the sunlight would have been great enough in other plants, and the exposure to the sunlight would probably have been long enough, at least for the *Fucus*. The penetration of the light rays into the tissues of seaweed must be much hindered by the thickness of the cuticle and it is conceivable that the light may be adequate for the process of carbon assimilation but not adequate or not of the right quality for the formation of vitamin A.

An exception, unexplainable at present, was found in the pollen of *Lilium candidum*. This contained large quantities of carotene (no xanthophyll by the phase test) which gave a typical spectrum and the usual lipochrome reactions. The ripe pollen from four stamens was rubbed on to a small piece of diet each day and given to each test rat. A week of this test gave very little growth and the pollen began to decompose; the remainder was pounded up with anhydrous sodium sulphate extracted with light petroleum and the extract evaporated down, mixed with a little inactivated olive oil and a drop of this solution (about 50 %) used as the daily ration. This resulted in no further growth and it was decided that the pollen was probably inactive, but the test will be repeated next summer (Table II).

The association of vitamin A with lipochromes in tissues not under the influence of light.

The classic example of this association is the root of the carrot, which has long been known to contain appreciable quantities of vitamin A. Its lipochromes are almost entirely carotene. The sweet potato is also reported by Steenbock as containing both carotene and vitamin A, and yet these two structures are almost completely in the dark, during their growing period. It does not seem probable that their subsequent exposure to variable and often very dull light after removal from the soil would bring about the formation of the relatively large quantities of vitamin A reported in these tissues.

A confirmation of this association in the carrot was made on the unsaponifiable fraction obtained in the usual way, and at the same time the corresponding fraction from turnips was prepared. The carrot extract used in a dosage of 0·01 g. produced very definite growth, the turnip extract produced none at all (Table II).

The question arose whether it was possible that the vitamin had been formed *in situ* in the root of the carrot or whether it had been transported

there from the leaves, where it is known to occur (earlier references). It is, of course, impossible to cut off the current of organic matter passing down to the root of the plant without at the same time preventing the development of the root; and, although not a strictly comparable process, it was decided to see whether the vitamin in the root could be transported upwards to leaves grown from carrots in the dark. Carrots were therefore cut off about an inch from the base, the cut ends placed in water and allowed to sprout. The leaves grew with very long stems, often 5 ins. long, and very small blades, never more than $\frac{3}{8}$ in. in diameter and mostly less. The age of these shoots could not be controlled as the age of the etiolated seedlings had been, so that the material used for the feeding test was from 3 to 5 weeks old. A dose of 0·1 g. of this fresh material made no impression on the growth of the test animals, but 0·2 g. produced a definite resumption of growth. A contrast to this result was obtained by testing 0·2 g. of the leaves of turnips sprouted in the dark: this dosage gave slight growth in one rat, none at all in three (Table II). The shoots of the carrot contained carotene, as shown by the spectrum, phase test, and lipochrome reactions, and no xanthophyll; the turnip shoots contained no lipochrome. Hence, it appears that the vitamin can be transported upwards at least, and when this occurs carotene seems to be transported also. Vitamin has been found in the green stems of polyanthus narcissus flowers (Tables II and III), but the stems contained lipochromes, the epidermis had stomata and assimilatory tissue and hence was comparable to the leaf of the plant. Hence, until more accurate investigation of the different regions of these stems has been carried out, it is not possible to draw any conclusion from the last observation.

The chief point in the formation of the vitamin which is apparent from these experiments is that some lipochrome (generally carotene) is always associated with the vitamin in plant tissues; and that where carotene is found, particularly carotene exposed to sunlight, there the vitamin may be expected to be present also.

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